Proceedings
of the
Society
for
Experimental Biology and Medicine

Vol. 63 November, 1946 No. 2

SECTION MEETINGS

MINNESOTA University of Minnesota	October 9, 1946
PACIFIC COAST University of California	October 2, 1946
ROCKY MOUNTAIN Colorado State College	October 19, 1946
SOUTHERN Louisiana State University	September 27, 1946

15555

Chemical Analysis of the C'l Fraction of Human Complement.*

E. E. Ecker, B. Lustig, A. A. Kondritzer, and S. Seifter.

From the Institute of Pathology, Western Reserve University, and the University Hospitals, Cleveland, Ohio, and the Research Laboratory of Lawrence Richard Bruce, Inc., Stamford, Conn.

Although the chemical and biological functions of the 4 components of human complement have been studied in considerable detail,[1-6] little is known of the composition and structure of the individual components. The preparation of an electrophoretically homogeneous midpiece (C'l) fraction has been reported.[3] In order to characterize the constitution of this fraction of human complement, a supply of electrophoretically homogeneous C'l was prepared and subjected to an elementary as well as an amino acid analysis.

Preparation. C'l was separated from human serum by the method described in a previous publication of this laboratory.[3] It was obtained as a β-globulin comprising 0.6% of the total serum protein. An ultracentrifuge study of this preparation disclosed the presence of a major component comprising 70% of the total fraction. Sedimentation constant of this component was 6.9 Svedberg units referred to water at 20°C. The remaining 30% of the fraction sedimented more rapidly.

On a dry basis the preparation contained 13.9% nitrogen and after repeated ether extraction contained 15.0% nitrogen, demonstrating that approximately 10% of the

---

* Aided by a grant from the Commonwealth Fund.

[1] Ecker, E. E., Pillemer, L., and Seifter, S., *J. Immunol.*, 1943, **47**, 181.

[2] Seifter, S., Pillemer, L., and Ecker, E. E., *J. Immunol.*, 1943, **47**, 195.

[3] Pillemer, L., Seifter, S., San Clemente, C. L., and Ecker, E. E., *J. Immunol.*, 1943, **47**, 205.

[4] Dozois, T. F., Seifter, S., and Ecker, E. E., *J. Immunol.*, 1943, **47**, 215.

[5] Seifter, S., Dozois, T. F., and Ecker, E. E., *J. Immunol.*, 1944, **49**, 45.

[6] Dozois, T. F., Seifter, S., and Ecker, E. E., *J. Immunol.*, 1944, **49**, 31.

original material was ether-soluble (lipoid). The ether-insoluble portion was subjected to analysis.

Methods of Analysis. Since analytical values are usually presented on the basis of ash-free material, the ash content of the ether-insoluble portion of C′l was determined. It was found to be 1.09% exclusive of NaCl.

Carbon, hydrogen, and total sulfur analyses were performed by the microchemical methods of Pregl, and were recalculated on an ash-free basis. For these determinations, samples were first brought to constant weight by drying *in vacuo* at 70°C over phosphorus pentoxide.

For the determination of arginine, histidine, cystine, serine, and threonine, 150 to 200 mg of the protein was hydrolyzed with 20% hydrochloric acid for 24 hours. The solution was then carefully brought to pH 5.5 with sodium carbonate and diluted to volume. After 24 hours the humin which formed was collected by centrifugation, washed twice with water and analyzed for nitrogen by the Kjeldahl micro-method. In the hydrolysate, arginine and histidine were determined colorimetrically at 490 and 515 mμ respectively, by the procedure of Macpherson.[7] Cystine was determined at 640 mμ by a similar method.[8] Serine was determined in the manner described by Nicolet and Shinn,[9] and the results were increased by 10% to allow for destruction during the acid hydrolysis.[10] The method of Shinn and Nicolet[11] was used to determine threonine. A Cenco-Sheard spectrophotolometer was used for all colorimetric determinations. All analyses were run in duplicate except for arginine.

For the analysis of tryptophane and tyrosine, 75 to 150 mg of protein were hydrolyzed with 5N sodium hydroxide for 24 hours. In the hydrolysate, tryptophane and tyrosine were determined by the Folin-Lugg method[12] at 490 and 515 mμ respectively. Tryptophane was also determined by the method of Eckert[13] at 550 mμ. Since the color in the Folin-Lugg method was observed to fade 45 seconds after the solution was mixed with the reagents, it is probable that Eckert's method is more reliable.

In connection with some other work, one of us (B.L.) has made a thorough investigation of the Tillmans-Alt orcinol reaction with carbohydrates. Experiments have shown that protein heated with 60% sulfuric acid gives a slight yellow color which absorbs light in the same region as does the color developed by the orcinol-carbohydrate reaction. The intensity of the color increases with the protein concentration.[14] However, if 0.2 ml of the 2% orcinol reagent is added to 60% sulfuric acid before heating with protein, the solution may be heated at 80°C for 20 minutes without producing any color which absorbs light at 520 mμ.

For the determination of polysaccharides in the C′l preparation, therefore, 0.4 ml of the ether-insoluble protein solution, 0.2 ml of orcinol reagent (2% in 25% sulfuric acid)

TABLE I.
Composition of the C′l Fraction of Human Complement (Per Cent of Ash-free Material.)

	%
Carbon	52.06
Hydrogen	7.59
Nitrogen	15.30
Humin-nitrogen	0.60
Cystine sulfur	0.64
Noncystine sulfur	0.55
Total sulfur	1.19
Arginine	4.66*
Histidine	1.87
Cystine	2.41
Methionine	2.09†
Serine	7.76
Threonine	11.18
Tyrosine	4.47
Tryptophane (Folin-Lugg)	1.08
(Eckert)	1.12
Polysaccharide	4.26

* One determination only.

† Calculated from noncystine sulfur, approximate only.

---

[7] Macpherson, H. T., *Biochem. J.*, 1942, **36**, 59.

[8] Lugg, J. W. H., *Biochem. J.*, 1932, **26**, 2160.

[9] Nicolet, B. H., and Shinn, L. A., *J. Biol. Chem.*, 1941, **139**, 687.

[10] Brand, E., Kassel, B., and Saidel, L., *J. Clin. Invest.*, 1944, **22**, 437.

[11] Shinn, L. A., and Nicolet, B. H., *J. Biol. Chem.*, 1941, **138**, 91.

[12] Lugg, J. W. H., *Biochem. J.*, 1937, **31**, 1422.

[13] Eckert, H. W., *J. Biol. Chem.*, 1943, **148**, 205.

[14] Heidelberger, M., and Kendall, F. E., *J. Immunol.*, 1936, **30**, 267.

and 6.0 ml of 60% (by volume) sulfuric acid were thoroughly mixed and heated for 20 minutes at 80°C. The intensity of the color in the cooled solution was measured at 520 mμ. For a blank sample, 0.4 ml of the same protein solution was mixed with 0.2 ml of distilled water and 6.0 ml of 60% sulfuric acid and treated as described above. The error of the method is ±2.0%. Determinations were made in duplicate.

Summary. A tabulation of the elementary, (partial) amino acid, and carbohydrate composition of the preparation of C'l is presented in Table I. The figure for hydroxy amino acid content of the C'l preparation (23.4%) compares favorably with the value of 21.3% obtained for a β-globulin concentrate by Brand, Kassel, and Saidel,[10] and with the value of 22.5% obtained by these authors for γ-globulin.

15556

Preparation and Properties of Erythrocyte-free Avian Plasmodia.*

LESLIE A. STAUBER AND HARRY A. WALKER. (Introduced by A. P. Richardson.)

From the Bureau of Biological Research, Rutgers University, and the Division of Pharmacology, The Squibb Institute for Medical Research, New Brunswick, N.J.

While studying the serological specificity of avian plasmodia using hemolyzed, parasitized erythrocytes as antigen and the rabbit for the production of antiserum we confirmed the experience of previous workers[1,2] that absorption of such antisera with uninfected erythrocytes seemed to remove all agglutinative or precipitative antibodies reactive with infective erythrocytes or extracts of them. It has been suggested that the malaria parasite may contain substances antigenically similar to normal blood cell components which would explain this phenomenon.[3-5] However, this suggestion does not explain the species and even strain specificity of malarial immunity in the vertebrate host though it may be an additional factor in the reaction. In order to account for the observed absorption of antibodies by uninfected erythrocytes Eaton and Coggeshall reasoned that the complicating factor was "that the parasites cannot be completely separated from the red cells of the monkey or other animals in whose blood they multiply."[1]

Our attempts to produce better test antigens by fragmentation (grinding or freezing and thawing) of duck red cells parasitized with *Plasmodium lophurae* and *Plasmodium cathemerium* were unsuccessful. Microscopically these preparations showed only a small proportion of objects which might be called intact free parasites whether the cells were exposed for a short or a long time to such mechanical treatment. It is possible that such ground materials would be poor test antigens since some precipitin reactions can be demonstrated only after adsorption of the antigen on collodion particles. Thus, the very integrity of the parasite may be essential to the best reactivity if only as a function of the surfaces involved. The removal of the plasma membrane of the erythrocyte in such a fashion as to free whole malaria parasites then seemed the first step in the experimental attack.

The nucleus of the avian erythrocyte served as one of the best clues to the in-

---

* This work was supported in part by the Protein Metabolism Fund of the Bureau of Biological Research.

[1] Eaton, M. D., and Coggeshall, L. T., *J. Exp. Med.*, 1939, **70**, 131.

[2] Zuckerman, A., *J. Infect. Dis.*, 1945, **77**, 28.

[3] Heidelberger, M., and Mayer, M. M., *Science*, 1944, **100**, 359.

[4] Oliver-Gonzalez, J., and Torregrosa, M. V., *J. Infect. Dis.*, 1944, **74**, 173.

[5] Butts, D. C. A., *Am. J. Trop. Med.*, 1945, **25**, 417.

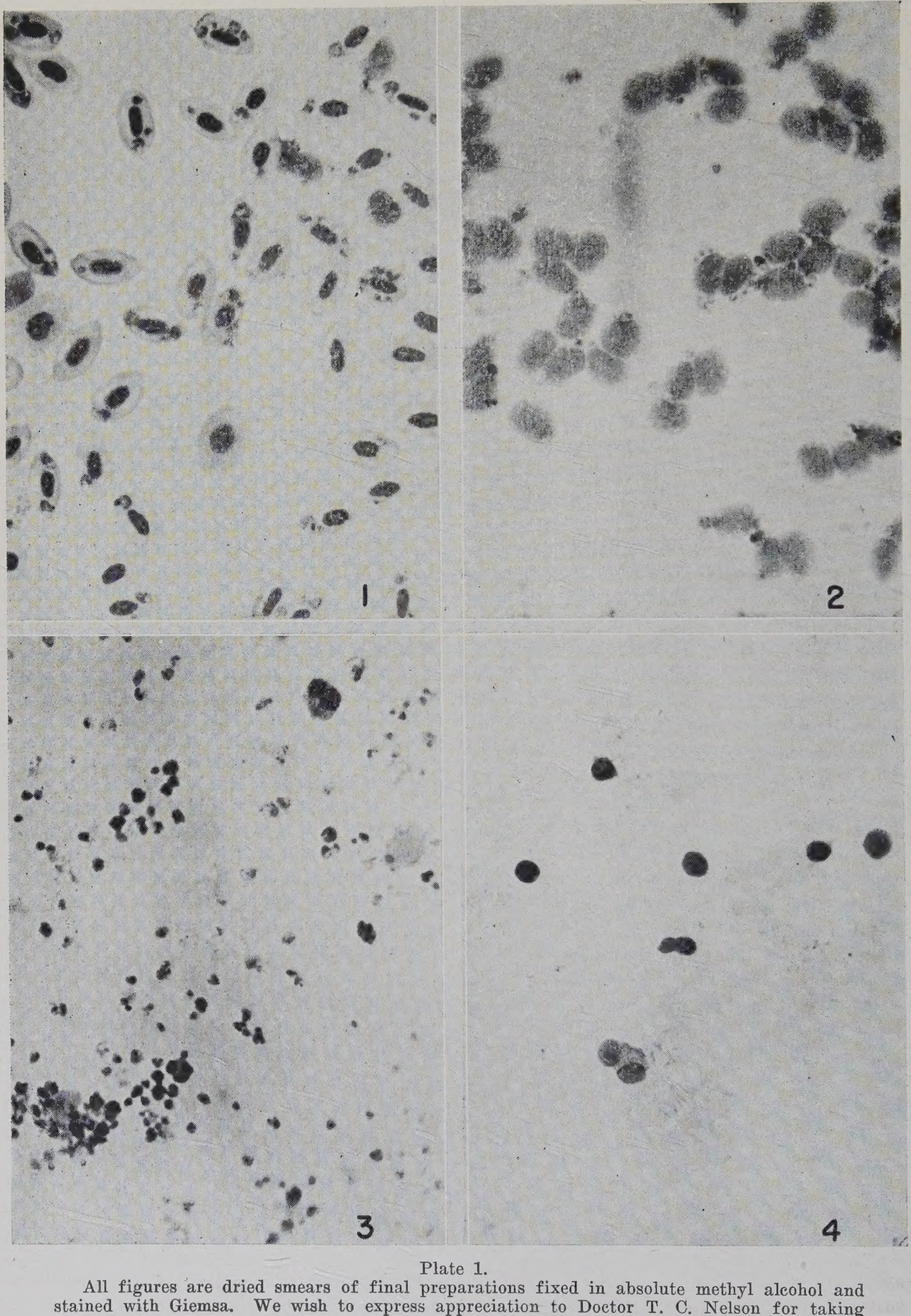


Plate 1.

All figures are dried smears of final preparations fixed in absolute methyl alcohol and stained with Giemsa. We wish to express appreciation to Doctor T. C. Nelson for taking the photomicrographs. Magnification approx. X1100.

Fig. 1.

Blood of a duck infected with *Plasmodium lophurae* exposed to all steps except hemolysis with saponin.

Fig. 2.

Infected blood exposed to all procedures except those containing enzymes.

Fig. 3.

Free parasites obtained by exposing infected blood to both hemolysis and enzymatic digestion.

Fig. 4.

Smear from end product of treatment of uninfected blood by both hemolysis and enzymatic digestion.

tegrity of the plasma membrane of the erythrocyte since it could be seen very easily in the fresh condition and was readily stainable by methylene blue or by the Romanowsky stains. It was not always possible to demonstrate satisfactorily the plasma membrane of the erythrocyte microscopically. Still, the presence of a parasite close to a nucleus and rigidly maintaining its position relative to that nucleus in a moving fluid medium was clearly indicative of the presence of at least a part of the stroma and was more than suggestive of the presence of the plasma membrane as well.

Although it is clearly indicated in Ponder's[6] summary that hemolysis is not synonymous with fragmentation or lysis of the stroma, the first attempts to free the parasites were made with hemolytic agents. Distilled water, chilled water saturated with CO_2, and various concentrations of NaCl, saponin, and urea were tried. Intact free parasites were not obtained by any of the procedures used. Even a technic successful in freeing *Plasmodium knowlesi*[7] from simian erythrocytes left *P. lophurae* still associated with the red cell nucleus of the duck.

Although living cells resist enzymatic digestion, Northrop[8] showed that intracytoplasmic introduction or proteolytic enzyme caused the disintegration of amebae. During hemolysis a change in the permeability of the erythrocyte occurs such that the large molecule of hemoglobin escapes from the cell.[9] If such altered permeability would permit the penetration of enzymes, cellular fragmentation with release of intact parasites might be accomplished. In the first trials, hemolysis by various agents followed by digestion with trypsin-steapsin mixtures at pH 7.0-7.8 resulted in failure to produce erythrocyte-free malarial plasmodia. However, when the enzyme preparations were tried at pH 5.0-5.5,[10] intact free parasites were obtained.

The method now used is as follows: Sterile parasitized duck blood is drawn into 0.9% saline solution, buffered to pH 7.0[11] and containing 300 mg % of heparin as anticoagulant. One part of the heparinized saline is used for every 19 parts of blood. All equipment and solutions except the saponin are sterile. After centrifugation at approximately 2000 r.p.m. for 10 minutes the plasma is drawn off and the cells washed once with 2-3 volumes of buffered saline. The cells may be washed at this point in the procedure with buffered saline to which formalin has been added in a concentration of 0.2%. In this way approximately 10 ml of packed cells are obtained for every 30-35 ml of duck blood drawn. The washed cells are suspended in 20 × their volume of 1-5000 saponin in buffered saline. The material is then centrifuged for 20 minutes and the supernatant drawn off. The cells are next suspended in 10 × their volume of Seitz-filtered enzyme solution prepared by dissolving 100 mg % of trypsin and steapsin in buffered saline (pH 5.0-5.5). The mixture is centrifuged immediately and the supernatant discarded. This step is necessary to remove any residual saponin. The cells are again suspended in

---

[6] Ponder, E., *The Mammalian Red Cell and the Properties of Haemolytic Systems*, Berlin, 1934.

[7] Christophers, S. R., and Fulton, J. D., *Ann. Trop. Med. and Parasit.*, 1939, **33**, 161.

[8] Northrop, J. H., *J. Gen. Physiol.*, 1936, **9**, 497.

[9] Davson, H., and Danielli, J. F., *The Permeability of Natural Membranes*, New York, 1943, 263-277.

[10] Ballentine, R., and Parpart, A. K., *J. Cell. and Comp. Physiol.*, 1940, **16**, 49.

[11] Evans, A. C., *J. Infect. Dis.*, 1922, **30**, 95.

10 × their volume of enzyme solution and allowed to stand with occasional shaking at room temperature for a total of 1½ hours, after the first addition of the enzyme. The material is then centrifuged and washed twice with buffered saline and finally made up to a total volume of 10 ml in buffered saline or buffered saline containing 0.2% formalin since with the latter we have found that the preparations keep well for at least several weeks when stored in the refrigerator.

Treated in this way uninfected duck cells lose all visible indications of hemoglobin and appear as a creamy mass of sedimented material in a volume usually equal to less than one-third the volume of packed cells at the beginning of the run. Prepared with erythrocytes heavily infected with *Plasmodium lophurae* (or other species of malaria) the material is muddy in appearance, the depth of the brown color and the volume is dependent upon the degree of parasitization of the erythrocytes.

It is not claimed that the empirical procedures used accomplish much digestion else there would not be the sediment seen with uninfected cells, but microscopically marked changes are evident. In wet mounts only granular material, sometimes in amorphous masses, and leucocytes are seen. It is especially interesting to note that the leucocytes seem entirely unchanged by the methods used. The end product with infected blood contains, in addition to the above, spherical parasites of a range of sizes from slightly more than 1 μ to almost 7 μ in dried smears. Granules of malaria pigment in active Brownian motion can be seen, usually more or less aggregated in a vacuole in one portion of the parasite.

When dried films of such preparations are fixed in absolute methyl alcohol and stained with Giemsa they appear as in Fig. 1-4. All smears were made from similar dilutions of original material and are as nearly comparable as possible. Fig. 1 is a smear of infected cells which have undergone exposure to all the steps except that of hemolysis in saponin. The parasites are practically normal in appearance and are largely contained within duck erythrocytes. Where outside of a cell they are closely adjacent to one of the paler nuclei, presumably belonging to an erythrocyte hemolyzed in the act of making the film. Note that in these cases a plasma membrane is not visible. Fig. 2 is from infected blood exposed to all procedures except those containing enzyme. The typical picture of stained hemolyzed red cells is seen. No plasma membranes are found and slightly swollen, paler nuclei are evident. Parasites are readily seen, usually pressed against the erythrocyte's nucleus and rarely more than a width of a cell away from the nucleus even though almost 50 parasites may be seen in the photomicrograph. Fig. 3 shows the result of hemolysis and digestion of infected duck blood. Aside from the leucocyte in the field and 2 smudges (incompletely altered erythrocytes) the field is full of free, intact malaria parasites. Their morphology is typical of that of the species to which they belong (in this case *P. lophurae*). The nuclei are somewhat larger and paler but the pigment and vacuolated cytoplasm are quite as seen in ordinary blood smears. *Plasmodium cathemerium* prepared in like fashion can be specifically distinguished from *P. lophurae* by the deeper, more homogeneous staining of the cytoplasm. Fig. 4 shows a field from the smear of uninfected blood after both hemolysis and digestion. Only leucocytes and pale smudges (possibly the remains of erythrocyte nuclei) are visible in the field. The leucocytes seem typical of those seen in ordinary thin films, one at least being obviously a heterophile.

Nonformalinized erythrocyte-free *P. lophurae* prepared as described above have been tested for infectivity by repeated intravenous injection of the material into 100 mg ducklings. Each duckling was injected with 1 ml of parasite suspension containing 3 billion parasites on alternate days for a total of 3 days. (Total dose 9 billion parasites per duck). The first injection was given on the same afternoon the concentrate was prepared and as soon after the last step in the procedure as possible. The material was then refrigerated and handled as if it were sterile until the last injection had been made. Only one of the 5 so treated showed parasites. It died on the 23rd day after the first injection with a parasitemia of 74%. This does not mean that parasites were able to survive the rigors of being freed from the erythrocyte by the means employed. We have repeatedly seen that small blood clots will protect the erythrocytes from hemolysis and therefore from digestion. Perhaps a single microscopic mass of such material could be infective and result in the death noted. We believe it most probable that the treatment destroys all those parasites freed from red cells.

Summary. By combining saponin hemolysis with enzymatic digestion at low pH avian plasmodia have been freed from the enclosing erythrocytes. These free parasites retain their specific morphology so that in stained smears of the erythrocyte-free parasites *P. lophurae* can be distinguished from *P. cathemerium.* It is probable that the treatment used kills the parasites but the potential value in experimental malariology of this new method is not necessarily reduced thereby.

15557

An Antibiotic (Anti-Smegmatis Factor) Produced by an Actinomycete, Specifically Inhibiting Species of *Mycobacterium*.*

Albert Kelner† and Harry E. Morton. (Introduced by A. N. Richards.)

From the William Pepper Laboratory of Clinical Medicine and the Department of Bacteriology, School of Medicine, University of Pennsylvania, Philadelphia, Penn.

The crude culture filtrate of an actinomycete (designated as A-82) has been observed to show extreme specificity in its action. It inhibited only species within the genus *Mycobacterium,* particularly the saprophytic species of *Mycobacteria,* and especially *Mycobacterium smegmatis.* This antibiotic will be referred to as the antismegmatis factor. The apparent genus specificity of the antismegmatis factor gives it unusual interest.

The actinomycete, A-82, was isolated from potted greenhouse soil by methods to be described elsewhere.[1] Although the organism resembles *Actinomyces lavendulae,* it differs sufficiently to justify a description.

Elliptical spores are borne in chains. Hyphae are coarse. No spirals were seen. Optimum temperature for growth about 28°C; growth at 14°C but not at 37°C. Peptone and gelatin media are blackened, but otherwise no soluble pigment is formed. Abundant grey growth on nutrient agar with white to grey spores. Thin spreading growth on Czapek medium with pink spores. Moderate growth on glucose asparagin agar with lavender colored spores. Good growth on calcium citrate agar; spores white, with trace of pink. Poor growth on calcium malate agar with white spores. Abundant grey-brown growth on potato with spores white, becoming lavender; the potato is blackened. Milk becomes alkaline with rapid clearing. Slow and variable liquefaction of gelatin. Moderate hydrolysis of starch.

The culture was maintained on the starch, potato, tryptone medium described by Kelner and Morton.[1]

Since *Mycobacterium smegmatis* (Univ. of Penn. strain P-49) was the organism most susceptible to the action of the antismegmatis factor, it was used for assay purposes. *M. smegmatis* was grown in Bacto-nutrient broth with daily transfers to stimulate rapid and diffuse growth. An 18-24 hour broth culture was used for streaking plates. The usually aggregated growth was dispersed by

---

* This investigation has been supported by a grant from the Smith, Kline, and French Laboratories.

† Present address, Carnegie Institute, Cold Spring Harbor, N.Y.

[1] Kelner, A., and Morton, H. E., in press.

TABLE I.
Production of Antismegmatis Factor by Actinomycete A-82.

Type of culture	Days 2	3	4	5	6	7	8	9	11	13
Stationary*										
pH of culture				6.4	6.8	6.6	6.2		7.0	
Activity†				400	400 (1000)	1000	1000 (4000)	1000	400 (1000)	
Stationary*										
pH of culture				6.6	6.8	6.8		7.0		
Activity†				4000	2000	2000 (4000)	4000 (10,000)	4000 (10,000)		2000 (4000)
Shake‡										
Activity†	40	40	40			1000 (2000)		2000 (4000)		
Shake‡										
pH of culture	6.2	5.8	6.0		4.8		4.6	4.2		
Activity†	40 (100)	400	2000		1000		200 (400)	400		

* Medium contained 30 ml glycerine/liter.

† *M. smegmatis* dilution units/ml (roughly equivalent to inhibiting dilution); one dilution unit being the smallest amount of crude culture filtrate per ml of assay medium which would inhibit *M. smegmatis* under conditions of the test. Figures in parenthesis indicate dilution of antismegmatis factor allowing only trace of growth of *M. smegmatis*.

‡ Medium contained 40 ml glycerine/liter.

vigorous shaking just before use, until the culture had a uniform, finely granular appearance. The agar dilution method of assay was used employing Bacto-nutrient agar, pH 7.3. *M. smegmatis* was streaked with a loop, and the plates read after incubation at 37°C for 18 to 36 hours, as soon as the control plate showed good growth. If the end-point was not sharp, it was helpful to dilute the culture 1½ to 2 times with sterile broth.

Titres of 2000 to 4000 (occasionally 10,000) *M. smegmatis* dilution units per ml of culture were formed when A-82 was grown in a medium composed of 5 g Bacto-tryptone, 30 ml glycerine, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.5 g KCl, 1.2 g Na_2HPO_4, 0.8 g KH_2PO_4, 0.01 g $FeSO_4 \cdot 7H_2O$, and 1000 ml distilled water; final pH, 6.8 to 7.2. For stationary cultures 2.5 g agar were added to the above formula. Incubation temperature was 28°C.

There was little or no antibacterial activity produced in nutrient broth or glucose-nutrient broth. In the tryptone-glycerine medium, the glycerine could not be replaced by dextrose, lactose, or brown sugar.

Table I shows the production of antismegmatis factor in stationary and in shake cultures.

Characteristics of the crude antismegmatis factor. Crude culture filtrates containing the antismegmatis factor, adjusted to pH 7.0, withstood, with little loss of activity, 56°C, for 45 minutes, and 100°C for 30 minutes. The material could be autoclaved at 121°C for 15 minutes with less than 50% loss of activity. Autoclaved material remained stable 6 days, and lost less than 50% activity in 10 days at 37°C. The crude antismegmatis factor was stored satisfactorily in the frozen state.

Partial concentration was easily accomplished by allowing frozen culture filtrates to thaw slowly with no disturbances. The bottom layer of the melted material contained 2 to 4 times more active substance than the top layer.

Intraperitoneal injection of 2 ml of crude culture filtrates (4000 *M. smegmatis* dilution units) had no apparent effect on white mice weighing 17 to 20 g.

The activity of the antismegmatis factor increases with the alkalinity of the assay agar, as can be seen in Table II.

TABLE II.
Relation of Alkalinity of Assay Agar to the Activity of the Antismegmatis Factor Against *M. smegmatis.*

pH	5.4	6.0	6.4	6.8	7.0	7.2	7.4	7.8	8.0	9.0
Activity*	10 (400)	20 (1000)	1000 (2000)	2000 (4000)	2000 (4000)	4000	4000	4000 (10,000)	10,000 (20,000)	20,000

*Dilution units, see footnote, Table I.

In acid media, and sometimes in neutral media, if the culture of *M. smegmatis* contained clumps, the assay plates showed a trace of growth at a wide range of dilutions, *e.g.*, from dilutions of 1:10 to 1:1000. The same phenomenon was shown by other sensitive organisms such as *M. phlei.*

A crude culture filtrate showing nearly complete inhibition of *M. smegmatis* in a dilution of 1:1280 was tested in Bacto-nutrient broth, pH 7.3, against the following microorganisms and found to have no inhibitory action at a dilution of 1:10: various species of *Bacillus, Brucella, Clostridium, Corynebacterium, Diplococcus, Eberthella, Escherichia, Micrococcus, Salmonella, Shigella, Staphylococcus,* and *Streptococcus,* as well as *Alcaligenes faecalis, Chromobacterium violaceum, Gaffkya tetragena, Klebsiella pneumoniae, Neisseria catarrhalis, N. sicca, Proteus vulgaris, Pseudomonas aeruginosa, Serratia marcescens, Trichophyton interdigitale* and *Vibrio comma.* It had very little if any inhibitory effect on a pathogenic strain of *Mycobacterium tuberculosis,* variety *bovis* (Ravenel strain) by the method described by Wells.[2]

A preparation containing 2000 dilution units failed to inhibit at a dilution of 1:10 in Bacto-nutrient agar *Candida albicans,* or in Czapek agar, *Aspergillus oryzae* or *A. niger.* In another experiment a preparation containing 2000 dilution units inhibited at a dilution of 1:10 but not at 1:20, *Bacillus subtilis,* and an unidentified penicillinase-producing *Bacillus* (No. 569 of the collection at Northern Regional Research Laboratory) at the same range of dilutions. *Mycobacterium phlei* (Univ. of Penn. No. P-50) was inhibited at a dilution of 1:400, with only a trace of growth at a dilution of 1:1000, and *M. tuberculosis* No. 607, a nonpathogenic strain‡ was inhibited at a dilution of 1:40, and showed only a trace of growth at 1:100 dilution, using 3% glycerine nutrient agar for the culture medium. In another experiment, using an antismegmatis factor preparation which inhibited *M. smegmatis* completely at 1:20,000, *M. tuberculosis* No. 607 showed only a trace of growth at dilutions of 1:20 to 1:20,000. The control showed abundant growth.

Discussion. The apparent genus specificity of the antismegmatis factor is of theoretical interest since it points to the possibility of antibiotics specific for other genera of bacteria or infectious agents. The specificity of the antismegmatis factor is probably not absolute, for more concentrated, chemically purified, preparations would probably inhibit such nonacid-fast organisms as *Bacillus subtilis,* an organism which was occasionally inhibited by very high concentrations of our most active crude culture filtrates. The far greater sensitivity of the *Mycobacteria,* as shown in our experiments, would still make the antismegmatis factor genus-specific. It is of course possible that genera not included in the 60-some strains of nonacid-fast organisms tested by us are sensitive to the antismegmatis factor. An example of great sensitivity of a single group of organisms for a compound is shown by yeasts for 2,3-dichloro-1,4-napthoquinone.[3]

Several *Mycobacterium*-inhibiting agents have been reported as being formed by

---

[2] Wells, W. F., *Science,* 1946, **104**, 254.

‡ We wish to thank Dr. Florence Seibert of the Henry Phipps Institute, Philadelphia, for this strain.

[3] Woolley, D. W., PROC. SOC. EXP. BIOL. AND MED., 1945, **60**, 225.

Aspergillus (usually *A. fumigatus*)[4-7] and by *Penicillium*.[8,9] Unfortunately little is reported of the bacterial spectra or other characteristics of these agents. However, "aspergillin"[7] may resemble the antismegmatis factor in that preparations inhibiting *M. phlei* at dilutions of 1:50 to 1:100 and *M. tuberculosis* at 1:10 to 1:20 failed to inhibit 2 strains of *Staphylococci*. Both "aspergillin" and "mycoidin"[6] are thermostable, as is the antismegmatis factor. Such *Mycobacterium*-inhibiting agents as streptothricin and streptomycin are clearly differentiated from the antismegmatis factor because they inhibit *Escherchia coli* and *Staphylococcus aureus* to the same degree they inhibit *Mycobacteria*.[10]

Of the older antibacterial agents, the most specific against *Mycobacteria* are the chaulmoogrates.[11] Hesse and Meisser[12] report that activity of several heterocyclic organic compounds against *M. tuberculosis* is correlated with their basic nature. The marked increase in activity of the antismegmatis factor with increasing pH, a property suggestive of a basic compound (see studies on streptothricin and streptomycin[13]) is of interest in this connection.

Although the antismegmatis factor failed to inhibit one strain of pathogenic *M. tuberculosis*, variety *bovis*, the fact that *M. tuberculosis* variety *hominis* No. 607 was inhibited, suggests that pathogenic strains of *M. tuberculosis* may be sensitive to preparations of the antismegmatis factor more concentrated than the crude culture filtrates we used and that there may be variations in sensitivity of strains within the species.

Summary. An actinomycete (A-82) forms an antibiotic which when tested against more than 60 species, including more than 20 genera of bacteria inhibited only *M. smegmatis*, *M. phlei*, and a nonpathogenic, rapidly growing strain of *M. tuberculosis*, variety *hominis* No. 607. A pathogenic *M. tuberculosis*, variety *bovis*, was not inhibited. The specificity of this antibiotic, referred to as the antismegmatis factor (because of the sensitivity of *Mycobacterium smegmatis*), for the genus *Mycobacterium* is discussed. Methods for producing the crude antismegmatis factor are outlined. The antibiotic is thermostable, and is more active in alkaline media than in acid media. No chemical purification of the antismegmatis factor was done.

---

[4] Asheshov, I. N., and Strelitz, F., *Science*, 1945, **101**, 119.

[5] Kallos, P., *Nature*, 1945, **155**, 300.

[6] Gerber, I. E., and Gross, M., *Science*, 1946, **103**, 167.

[7] Soltys, M. A., *Nature*, 1944, **154**, 550.

[8] Miller, D. K., and Rekate, A. C., *Science*, 1944, **100**, 172.

[9] Smith, M. I., and Emmart, E. W., *Pub. Health Rep.*, 1944, **59**, 417.

[10] Schatz, A., and Waksman, S. A., Proc. Soc. Exp. Biol. and Med., 1944, **57**, 244.

[11] Walker, E. L., and Sweeney, M. A., *J. Inf. Dis.*, 1920, **26**, 238.

[12] Hesse, E., and Meissner, G., *Arch. Exp. Path. u. Pharmakol.*, 1931, **159**, 676.

[13] Waksman, S. A., *Microbial Antagonisms and Antibiotic Substances*, 1945, The Commonwealth Fund, N.Y.

15558

The Biological Activity of Calciferol (Vitamin D_2).*

Aaron Arnold. (Introduced by M. L. Tainter.)
From the Sterling-Winthrop Research Institute, Rensselaer, N.Y.

The international unit of vitamin D is the activity of 1 mg of the international Standard of Reference which, in turn, is defined in terms of a solution of irradiated ergosterol in olive oil. Collaborative study[1] in 1940 showed

---

* The writer wishes to express his appreciation to Mrs. Ellen Miller for technical assistance and to Mrs. Mary Lenz for the statistical treatment of the data.

[1] Coward, K. H., *Bull. Health Org., League of Nations*, 1940-1, **9**, 425.

that the international unit represents the activity of 0.025 μg of pure crystalline calciferol, *i.e.*, 1 μg is equivalent to 40 international units. The U.S.P. unit of vitamin D, by definition,[2,3] is equivalent to an international unit. However, recently we have observed that calciferol[†] (vitamin D_2) exhibits greater activity than 40 U.S.P. units per μg when compared against the U.S.P. reference standard. This appears to be evidence that the present U.S.P. reference cod liver oil (No. 2) contains less vitamin D than would be expected from the original assay values.[4] If this observation is confirmed generally the activity of the U.S.P. standard should be reconsidered. There is considerable importance attached, therefore, to examining the data regarding the standard currently in use.

Experimental. Samples. The samples of calciferol tested were from current commercial production and were of a purity comparable to that described in an earlier publication from these laboratories.[5] The physical constants used as a check on the purity of the synthesized vitamin were melting point, specific rotation, and specific absorption.

For the tests, 20 to 30 mg of the samples were dissolved in corn oil and diluted so as to supply in 6 days approximately 5.0, 7.5, 8.0, or 8.65 U.S.P. units of vitamin D per rat.

Method of testing. The diluted calciferol samples were assayed by the U.S.P. XII procedure.[3] The test animals were from our own stock colony so that the healing response to the standard or test doses was moderately uniform. Groups of 8 to 10 animals were fed the vitamin D supplements for 6 successive days and were killed on the 8th day in accordance with the provisions of the U.S.P. method. Rats that did not gain weight properly were discarded.

The U.S.P. reference cod liver oil is fed routinely at 2 levels in our laboratory, while the test materials are usually fed at one level. Such tests lend themselves best to graphic analysis which method has been used for the potency estimations of single-level assays reported in this study. For tests which are designed to supply an estimate of potency along with an estimate of the standard error it is often preferred to test the unknown material at 2 levels. Under these conditions, any of several methods may be used to calculate the standard error of the determination, the one by Miller, Bliss and Braun[6] being used here. Potency estimates were calculated by the method of Bliss and Marks.[7]

Results. The results of the 1- and 2-level assays of the test calciferol samples are given in Table I.

It will be observed (Col. 8) that the calciferol samples were diluted on the assumption, based on past experience, that they would supply 49 U.S.P. units per μg; this assumption has no effect on the outcome of the test, of course, but must be mentioned to clarify the procedure used.

It will be seen that the single-level assays uniformly indicated slightly over 50 units per μg. The 2-level assays, Nos. 470 and 471, show potencies very close to 50 units per μg for all lots except Lot 60; the value for this sample by the 2-level assay (No. 471) was 46.5 ± 4.7 units per μg which is not significantly different from the value of 50.7 units per μg (Assay No. 458) obtained by the single-level assay on the sample.

Discussion. The data presented here leave no doubt as to the consistency with which these calciferol samples have shown more than 40 U.S.P. units per μg. The weighted mean of the 8 results of the 2-level assays is 49.7 with a weighted average standard error of ± 2.1 U.S.P. units per μg (calculated by the method of Miller, Bliss, and Braun.[6])

---

[2] Nelson, E. M., *The Vitamins,* A symposium, Chicago, 1939, 475-81.

[3] *U. S. Pharmacopoeia* XII, Easton, 1942, 635.

[†] Crystalline vitamin D_2 is most conveniently termed calciferol to distinguish it from Viosterol which represents irradiated ergosterol. The latter mixture contains vitamin D_2, unchanged ergosterol, and under—and over—irradiation products.

[4] U.S.P. Vitamin Advisory Board, *J. Am. Pharm. Assn.,* 1942, **31**, 124.

[5] Huber, W., and Barlow, O. W., *J. Biol. Chem.,* 1943, **149**, 125.

[6] Miller, L. C., Bliss, C. I., and Braun, H. A., *J. Am. Pharm. Assn.,* 1939, **28**, 644.

[7] Bliss, C. I., and Marks, H. P., *Quart. J. Pharm. and Pharmacol.,* 1939, **12**, 182.

TABLE I.

Biological Activity of 8 Lots of Calciferol Tested at One- and Two-levels of Assay at 6 Different Times.

	U.S.P. reference oil				Calciferol					
			Healing score					Healing score		
Test No.	No. of rats	Dose per rat U.S.P. units	Range	Mean	Lot No.	No. of rats	Dose per rat U.S.P. units	Range	Mean	Potency* U.S.P. units per μg
457	7	5.0†	1.5-1.75	1.64	59	9	8.65‡	1.75-3.0	2.28	51.6
	8	8.65	1.75-3.0	2.22	62	9	8.65	1.75-3.0	2.31	53.7
458	9	5.0	1.5-1.75	1.57	60	9	8.65	1.75-3.0	2.22	50.7
	9	8.65	1.75-3.0	2.19						
459	8	5.0	1.0-2.0	1.66	61	8	8.65	1.50-3.0	2.43	50.7
	7	8.65	1.75-3.0	2.39	63	7	8.65	1.75-3.0	2.43	50.6
					64	8	8.65	1.75-3.0	2.43	50.6
470	9	5.0	1.5-1.75	1.58	94	8	5.0	1.0-1.75	1.61	50.9 ± 5.9
	10	7.5	1.5-2.0	1.80		9	7.5	1.5-2.0	1.80	
					59	7	5.0	1.5-1.75	1.71	50.7 ± 5.4
						9	7.5	1.75-2.75	2.05	
					60	5	5.0	1.50-1.75	1.65	46.5 ± 4.7
						8	7.5	1.75-2.5	1.97	
					61	8	5.0	1.50-2.0	1.72	49.3 ± 5.9
471	6	5.0	1.5-1.75	1.67		8	7.5	1.75-2.75	2.00	
	6	7.5	1.75-2.5	2.04	62	5	5.0	1.50-2.0	1.75	51.4 ± 6.8
						7	7.5	1.75-2.75	2.04	
					63	7	5.0	1.50-1.75	1.71	51.1 ± 6.5
						7	7.5	1.50-2.75	2.07	
					64	5	5.0	1.50-1.75	1.65	49.6 ± 5.9
						5	7.5	1.75-2.75	2.08	
476	8	5.0	(all 1.75)	1.75	103	8	5.0	(all 1.75)	1.75	49.4 ± 7.8
	8	8.0	1.75-2.5	1.97		8	8.0	1.75-2.5	1.98	
						Mean (2-level assays)				49.7 ± 2.1

---

* For the estimation of potency of the calciferol samples tested at a single level (assay Nos. 457, 458 and 459), a curve was constructed (healing vs. log dose) on the basis of the 2 levels of the reference oil standard. The average healing score for each sample was converted to units of biological activity on the basis of the straight line curve of the respective assay.

The potency estimates of the 2 level assays were calculated statistically in accordance with the method of Bliss and Marks[7] from the potency formula $M = \frac{ID}{B}$ where M = antilog of the estimated potency of the unknown as compared with the standard; I = difference between the logarithms of the doses; D — difference in the responses to the sample and to the standard; B = difference in response between levels for the sample and the standard.

In view of the fact that all groups did not have the same number of animals (some of the rats could not be used since they did not gain sufficient weight during the test period or showed "starvation healing"), the mean healing scores were substituted for the missing values in order to bring the groups up to equal size. This does not affect the calculated standard error significantly as long as fewer than one-quarter of the total observations for each calculation are substituted values.

† The U.S.P. reference cod liver oil was diluted for test on the basis of 115 U.S.P. units per g in accordance with the label statement. Several bottles of the reference oil were used in the course of these studies.

‡ The calciferol samples were diluted for test on the assumption that they would supply 49 U.S.P. units per μg.

This result obtained on different lots of calciferol indicates that, as currently produced, calciferol has a potency much greater than 40 of the present U.S.P. units per μg. The error of the assay is such that no one sample can be said to be significantly more potent than 40 U.S.P. units per μg, *i.e.*, the odds are only about 1 in 10 in support of such a conclusion. However, taken together and considering all samples as representative of current commercial production, the average indicates that the odds are only 1 in 1000 that the value of 49.7 U.S.P. units per μg is a chance occurrence. These results agree closely with those obtained in a recent collaborative study on vitamin D_3 with chicks;[8] the grand average value compiled by Waddell therein indicated the latter vitamin to have slightly over 49 A.O.A.C. units per μg. Since the A.O.A.C. unit and the U.S.P. unit are equivalent by rat assay,[9] additional and independent evidence is thus at hand to indicate that vitamin D_3, as well as calciferol, assays at more than 40 U.S.P. units per μg.

As indicated above, a collaborative study carried out some years ago (in which 9 laboratories participated) showed that calciferol[‡] has a potency of 40 international units per μg. Since the physical constants of our calciferol do not appear to be different from those given for the material used in the study reported by Coward,[1] ours should not show 25% greater activity. We must turn to the alternative explanation that the current U.S.P. reference cod liver oil is less potent than the original international standard. The U.S.P. unit must be defined in terms of the U.S.P. reference standard to satisfy legal requirements. Thus it cannot be said that the U.S.P. unit is too small. It does appear clear, however, that at present one U.S.P. unit has only 0.8 the activity exhibited by 1 mg of that international standard which some years ago was found equivalent to 0.025 μg of calciferol.

Brief mention may be made of the findings of Gridgeman[11] that pure calciferol showed only 35.7 international units of vitamin D per μg while vitamin D_3 was equivalent to 46.6 international units per μg. This observation that vitamin D_3 is 1.31 times as potent as calciferol is so markedly at variance with earlier data obtained in the extensive collaborative studies reported by Coward[1] that independent confirmation is needed. The data presented in this communication and in the A.V.R.C. report[8] based on chick assays, agree in showing that both calciferol and vitamin D_3 assay 49 to 50 U.S.P. units per μg when referred to the U.S.P. or A.O.A.C. standard (these are equivalent[9]). These data are interpreted as showing a 1:1 ratio of the potencies.

Summary. Assays of 8 samples of calciferol (vitamin D_2) using the U.S.P. biological assay with rats indicated in every case a potency significantly greater than 40 U.S.P. units per μg. Our assays gave an average value of 49.7 ± 2.1 units per μg. This is interpreted to mean that U.S.P. reference cod liver oil No. 2 has less than its generally accepted potency. In view of this finding, a re-evaluation of the relationship between calciferol, the international standard and the U.S.P. standard for vitamin D would seem to be indicated. Serious consideration should be given to adopting a fixed weight of pure crystalline calciferol or vitamin D_3 as the legal unit instead of relying on designated weights of reference standards consisting of solutions of impure and possibly unstable mixtures.

---

[8] News Item, *Chem. Eng. News*, 1944, **22**, 2053.

[9] Assn. Official Agr. Chem., *Official and Tentative Methods of Analysis*, 5th ed., Washington, 1940, 372.

[‡] The international standard is not calciferol in oil as stated by Reed, Struck, and Steck.[10]

[10] Reed, C. I., Struck, H. C., and Steck, I. E., *Vitamin D: Chemistry, Physiology, Pharmacology, Pathology, Experimental and Clinical Investigations*, Chicago, 1939, 58.

[11] Gridgeman, N. T., *Quart. J. Pharm. and Pharmacol.*, 1945, **18**, 24.

15559

Swelling of Brain Slices and the Permeability of Brain Cells to Glucose.

K. A. C. Elliott.

From the Department of Neurology and Neurosurgery, McGill University, and the Montreal Neurological Institute, Montreal.

Sperry and Brand[1] found that the percentage dry weight of rat liver slices immersed in physiological saline solutions decreased rapidly for 15 minutes and then more slowly. They concluded that the effect was due to absorption of water by the slices. The observations of Sperry and Brand have been confirmed, though somewhat smaller effects than theirs were obtained. But by weighing the slices before and after immersion, which the previous authors did not do, it was found that the decrease in percentage dry weight of liver slices is largely due to leaching of solid matter out of the slice and that the actual swelling or increase in weight amounts to only about 10%.

Rat brain cortex slices in isotonic saline solutions swell more than this and some factors affecting such swelling are here described.

Methods. The slices were prepared in a humid chamber similar to that described by Sperry and Brand.[1] It was illuminated by a lamp behind a trough of water. The bottom of the chamber was covered with several layers of blotting paper which were thoroughly moistened with water, and air was passed through an aquarium aerator stone immersed in a beaker of water in the chamber. (A glass shield against the beaker prevented spray from falling on the tissue). By this method the humidity was kept at approximately 100%, judging by equal readings of wet and dry bulb thermometers, and the high and uneven temperatures, and the dripping of condensed vapor, which occur when the humidity is produced by hot water, were avoided.

Uniform slices of brain cortex were cut by means of a Stadie and Riggs[2] microtome designed to cut slices about 0.45 mm thick. Four surface slices, each weighing 45 to 75 mg, were cut from each brain. Thoroughly satisfactory slices were obtained without moistening the blade or microtome provided the surfaces of these were wiped clean after cutting each slice. The slices were spread on a watch glass in the chamber until used and then weighed outside on a torsion balance.*

Individual weighed slices were immersed in small beakers containing the fluids to be tested and these were aerated continuously with oxygen or, in the case of bicarbonate-containing fluids, with oxygen-5% carbon dioxide mixture. All media contained at least 0.2% of glucose. After immersion the slices were drained on a glazed perforated disc, spread onto hardened filter paper (Whatman No. 50) gently wiped with torn edges of soft filter paper, removed from the paper, and weighed. This procedure involved an appreciable loss of weight, which was estimated on control slices which were immersed in saline solutions for a few seconds. Experimental slices were immersed for 20 minutes; longer immersion gave relatively little further changes. The fluids were in general kept at room temperature since tests at 38° gave similar results.

Results. Significant results are summarized in Table I and Fig. 1. Concentrations of solutes are expressed in terms of isotonicity with an 0.308 osmolar solution such as normal saline. All figures for percentage swell-

---

[1] Sperry, W. M., and Brand, F. C., Proc. Soc. Exp. Biol. and Med., 1939, **42**, 147.

[2] Stadie, W. C., and Riggs, B. C., *J. Biol. Chem.*, 1944, **154**, 687.

* This procedure whereby uniform slices are prepared without moistening has been used here (see also Fuhrman and Field,[3]), and is recommended for preparing slices for metabolism studies since it allows calculation of results on the basis of true, directly determined tissue weight.

[3] Fuhrman, F. A., and Field, J., 2nd, *J. Pharm. and Exp. Therap.*, 1943, **77**, 229 and 392; *Am. J. Physiol.*, 1943, **139**, 193; *J. Cell. Comp. Physiol.*, 1943, **21**, 307; *J. Neurophysiol.*, 1944, **7**, 117.

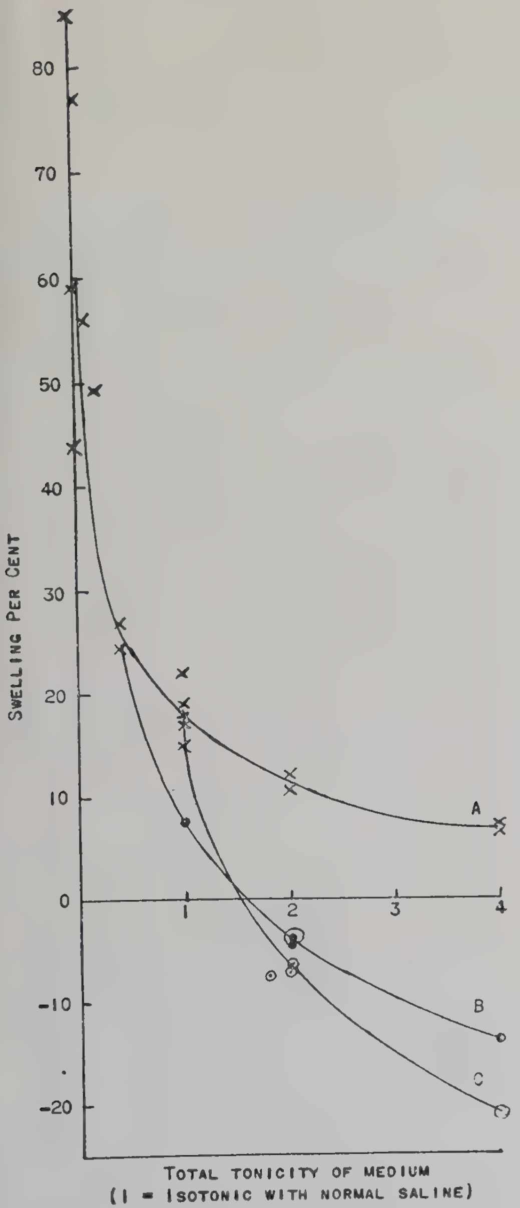


Fig. 1.

Effects of glucose and sodium chloride on the swelling of brain cortex slices.

Curve A—Tonicity made up with NaCl alone.

Curve B—0.4 isotonic NaCl with glucose added to give total tonicity indicated.

Curve C—1.0 isotonic NaCl with glucose added to give total tonicity indicated.

ing or shrinkage, that is increase or decrease in weight, are corrected for the average loss of weight of control slices. This loss varied between 5 and 12%, and in 9 experiments averaged 8%. (Determinations of dry weight on some slices, directly and after brief immersion, indicated that the percentage dry weight of the substance lost on brief immersion and draining was roughly the same as that of fresh tissue. After 20 minutes immersion little or no more dry substance was lost).

In water or pure sugar solutions the swelling of slices was very pronounced and the jelly-like consistency of the tissue prevented accurate drainage and determination of the extent of swelling. With increasing salt concentration the swelling decreased, but in isotonic sodium chloride solution the swelling still averaged about 18%, and even in 4 times isotonic solution some swelling occurred (Fig. 1, Curve A). The extent of swelling was about the same in isotonic sodium chloride, Ringer, Ringer-phosphate or Ringer-bicarbonate solution or in human spinal fluid. It was not affected by additions of manganese or cobalt (2×10^{-4}M). In isotonic potassium chloride the swelling was a little greater, and in isotonic calcium or magnesium chloride it was lower.

In horse or rat serum, the swelling was appreciably lower than in saline. Addition of gelatin,† as colloid material, to Ringer solution reduced the swelling appreciably only if large amounts were used.

The behavior or nonelectrolytes was of special interest. In isotonic (0.308 M) glucose, fructose or sucrose solutions, the tissue swelled as though it were in water, and the swelling was very marked even in 4 times isotonic solutions. However, in the presence of some sodium chloride, the effect of the sugars in decreasing swelling was even greater than that of the salt (Fig. 1, Curves B and C). The potentiating effect of salt is apparent when a glucose solution contains only 0.1 isotonic sodium chloride, and is almost maximal with twice this amount (Table I). The effect seems to be common to all electrolytes since equi-osmolar concentrations of sodium chloride, bromide, sulfate and phosphate, and potassium chloride gave similar results.

It would appear that the tissue cells are

---

† Knox P-20 Gelatine Solution, kindly supplied by Dr. D. Tourtelotte, Knox Gelatine Products, Inc.

TABLE I.

Percentage Swelling (Increase in Weight) of Brain Slices in Various Media.[*]

Medium[†]	% swelling[‡]	Medium[†]	% swelling[‡]
Water[†]	44, 59, 101, 77	Isotonic glucose	65
Isotonic NaCl	15, 19, 17, 22, 18	2 × Isotonic glucose	41, 25
Ringer sol.	16, 17, 14, 18, 17, 15.5, 17.5, 18	2 × " fructose	58
Spinal fluid (human)	15.5, 17	2 × " sucrose	50
Ringer + 1% gelatin	15.5, 15	4 × " glucose	33, 36
" + 3% "	9.5, 6	1.9 " " + 0.1 Isotonic NaCl	11.5
Serum (horse)	7, 7.5, 7	1.8 " " + 0.2 " "	—2
" (rat)	7	1.6 " " + 0.4 " "	—4, —4.5
Isotonic KCl	24	1.3 " " + 0.7 " "	—6.5
" $CaCl_2$	5	1.6 " " + 0.4 " Na_2SO_4	—4.5
" $MgCl_2$	4.5	1.6 " " + 0.4 " NaBr	—3
		1.6 " " + 0.4 " KCl	—4.5
		1.6 " " + 0.4 " Na phos. buffer	—8.5
		1.6 " fructose + 0.4 " NaCl	—6.5
		1.6 " sucrose + 0.4 " NaCl	—11.5

[*] This table does not include some of the results illustrated in Fig. 1.

[†] All media contained at least 0.2% glucose.

[‡] All figures have been corrected by the addition of 8, which is the average percentage weight lost on brief immersion and draining. Negative figures indicate shrinkage (loss of weight).

partially impermeable to electrolytes so that solutions of these can oppose swelling by osmotic action, though they cannot, even in high concentration, produce shrinkage. In the absence of electrolyte, the tissue behaves as though it is almost completely permeable to the sugars, while in the presence of even small amounts of electrolyte it becomes almost completely impermeable to sugars.[‡] Danielli[5] had discussed physical chemical factors concerned in effects of this type observed with red cells and *Arbacia* eggs. As is indicated in Fig. 1, slices can be maintained without appreciable swelling or shrinkage in a somewhat hypertonic solution consisting of Ringer or normal saline solution containing 0.15 M glucose.

The greater part of the swelling of slices in saline solutions isotonic with serum or stronger, seems to be due to intracellular colloidal osmotic pressure which can be balanced by serum proteins. If this is so it would indicate that the normal intercellular environment of brain cells is not simple spinal fluid but a protein-containing lymph. Even in serum some swelling occurs which is probably a result of damage due to cutting of cell processes or to effects on cell membranes of temporary anoxia or deprivation of circulation during the preparation of slices.

Small additions of materials known to affect permeability *in vivo* or nervous activity, namely adrenal cortical extract (10% of Connaught Laboratory Preparation), acetylcholine chloride and/or eserine (0.1%), metrazol (0.2%) or sodium pentobarbital (0.2%), did not significantly affect the swelling in isotonic sodium chloride solution or in a solution, containing 0.1 isotonic salt and 0.9 isotonic glucose, in which the permeability relations are delicately balanced.

Summary. 1. Brain slices swell considerably in salt solutions isotonic with serum and appreciably even in 4 times isotonic solutions. In serum the swelling is less marked.

2. Brain tissue behaves as if freely permeable to glucose, fructose and sucrose in the absence of electrolyte but, in the presence of small amounts of salt, these sugars behave as impermeable solutes.

[‡] In previous work[4] it was concluded, from studies on the respiration of brain tissue homogenized in various media, that brain tissue cells are impermeable to glucose and sucrose. This conclusion was probably correct for the conditions of the experiments concerned in which all media contained some electrolyte, 0.017 M phosphate, and a certain amount of other salts contributed by the tissue in the strong suspensions used.

[4] Elliott, K. A. C., and Libet, B., *J. Biol. Chem.*, 1942, **143**, 227.

[5] Danielli, J. F., in Davson, H., and Danielli, J. F., *The Permeability of Natural Membranes*, New York, 1943, p. 324.

15560

Streptomycin and Diasone in the Treatment of Experimental Tuberculosis of Guinea Pigs.

Fritz T. Callomon, John A. Kolmer, Anna M. Rule, and Albert J. Paul.
From the Research Institute of Cutaneous Medicine, Philadelphia, Pa.

Streptomycin has yielded encouraging results in the treatment of experimental tuberculosis of guinea pigs[1-3] and of Swiss mice,[4] especially by exerting a suppressive effect upon the development of lesions when administered within a few days to 2 weeks after inoculation with virulent tubercle bacilli. Varying dosage has been employed but daily total doses of 5000 to 6000 units in saline solution per guinea pig (approximately 10,000 to 15,000 units per kg), divided into 4 to 6 subcutaneous or intramuscular injections, have been found satisfactory and generally well tolerated. Various derivatives of diamino-diphenylsulfone have also been found somewhat effective in the treatment of experimental tuberculosis of these animals.[5,6]

The purpose of our experiments was to determine not only the therapeutic effectiveness of streptomycin in experimental tuberculosis of guinea pigs, but the possible synergistic or additive effects of combined streptomycin and diasone (a diamino-diphenylsulfone derivative) therapy in this disease.

In the first experiment 20 guinea pigs varying in weight from 320 to 485 g were inoculated subcutaneously with 0.5 mg of culture H42733 of human tubercle bacilli per 100 g body weight* Ten were kept as untreated controls and 10 subjected to streptomycin treatment beginning on the 3rd day after inoculation.† The dose of the compound per animal was 1500 units in saline solution, by subcutaneous injection, at 9:30 a.m. and 1:30 p.m. with an intramuscular injection of 6000 units suspended in sterile peanut oil and 3% beeswax at 4:30 p.m., totalling 9000 units per animal per day. After 10 to 14 days of treatment, however, many of the animals showed evidences of toxic effects of the compound and there were 2 deaths. Under the circumstances treatment was interrupted for 4 days and then resumed with a total daily dose of 5000 units which was raised to 6000 units beginning with the 4th week of treatment and continued for a total of 72 days after inoculation; all surviving treated and untreated animals were sacrificed for autopsy examinations. Microscopic and macroscopic examinations of the spleen, liver and lungs were made of animals succumbing during the experiment or sacrificed at its termination. Assays of the serum in a number of animals showed about 6.5 units of streptomycin per cc 20 minutes after the subcutaneous injection of 1500 units in saline solution, which dropped to about 2.5 units per cc at the end of 1 hour and to between 1-2 units at the end of 2 hours. Two hours after intramuscular injections of 3000 units in peanut oil and 3% beeswax no streptomycin could be detected in the serum with 1 to 2 units per cc at the end of 4 hours and none at the end of 6 and 18 hours. It would appear, therefore, that streptomycin suspended in peanut oil and beeswax is not absorbed as readily and uniformly from the muscles of guinea pigs as penicillin. Similar results have been observed in human adults following single intramuscular injections of 250,000 and 500,000 units of streptomycin

---

[1] Feldman, W. H., and Hinshaw, H. C., *Proc. Staff Meet. Mayo Clinic*, 1944, **19**, 594.

[2] Feldman, W. H., Hinshaw, H. C., and Mann, F. C., *Am. Rev. Tuberc.*, 1945, **52**, 269.

[3] Smith, M. I., and McCloskey, W. T., *Pub. Health Rep.*, 1945, **60**, 1129.

[4] Youmans, G. P., and McCarter, J. D., *Am. Rev. Tuberc.*, 1945, **52**, 432.

[5] Feldman, W. H., Hinshaw, H. C., and Moses, H. E., *Am. J. Med. Sc.*, 1944, **207**, 290; *Arch. Path.*, 1944, **36**, 64.

[6] Callomon, F. T., and Raiziss, G. W., *Am. Rev. Tuberc.*, 1946, **53**, 374.

* Kindly supplied by the Phipps Institute, Philadelphia, Pa.

† Streptomycin sulfate kindly supplied by the Abbott Laboratories, North Chicago, Ill.

TABLE I.
Streptomycin in Treatment of Experimental Tuberculosis of Guinea Pigs.

	No.	Duration of life (days after inoculation)	Results of autopsies[*]		
			Spleen	Liver	Lungs
Treated	1	Killed 72 days	+	—	+
	2	” ” ”	+	—	++
	3	” ” ”	+	—	++
	4	” ” ”	—	—	+
	5	” ” ”	+++	—	++++
	6	Died 19 ”	—	++	+++
	7	Killed 72 ”	+	+	+++
	8	Died 15 ”	++	—	—
	9	Killed 72 ”	++	+	++
	10	Died 37 ”	++	+	++++
Controls	11	Died 56 ”	++	+++	++++
	12	Killed 72 ”	++	—	++++
	13	” ” ”	+++	—	++++
	14	” ” ”	+++	—	+++
	15	Died 25 ”	—	++	—[†]
	16	Killed 72 ”	++	++	++++
	17	” ” ”	++	++	++++
	18	” ” ”	+++	—	+++
	19	” ” ”	+	+++	++++
	20	” ” ”	+++	+	+++

[*] — = no lesions; + = occasional small tubercles or one small solitary tubercle; ++ = many microscopic tubercles; +++ = moderate involvement showing focal rather than diffuse tuberculosis with or without necrosis; ++++ = severe progressive tuberculosis with necrosis.

[†] Acute nontuberculous pneumonia.

suspended in peanut oil and 4% beeswax.[7]

The results are shown in Table I and may be summarized as follows: (a) of 10 untreated controls 2 succumbed; (b) of 10 treated animals 3 succumbed although 2 of these deaths (Nos. 6 and 8) were thought to be due to the toxicity of streptomycin in the high initial dosage of 9000 units per animal per day; (c) all of the treated animals presented tuberculous lesions at autopsy but their incidence and severity were somewhat less than observed among the untreated controls and indicative of some suppressive therapeutic activity on the part of the streptomycin in the doses and method of administration employed.

In the second experiment 32 guinea pigs, weighing 350 to 500 g, were inoculated subcutaneously with 0.75 mg of the same culture per 100 g of weight in order to produce more severe infections, and treatment was not instituted until 18 days after inoculation. One group of 8 animals was then given 1500 units of streptomycin in saline solution by subcutaneous injection at 9:30 a.m. and 1:30 p.m., and 3000 units in peanut oil and 3% beeswax intramuscularly at 4:30 p.m., totalling 6000 units per day for 32 days. A second group of 8 animals were given about 210 to 240 mg of diasone[‡] containing 10% sodium bicarbonate as a stabilizer and equivalent to about 190 to 215 mg of pure diasone in food per day for 32 days. A third group of 8 animals were given a similar daily dose of diasone for 32 days, along with injections of streptomycin which were started on the 4th day of treatment, covering a period of 28 days. A 4th group of 8 animals were kept as untreated controls. All animals succumbing during the experiment were subjected to macroscopic and microscopic examinations of the spleen, liver and lungs for tuberculous lesions and all surviving the total of 50 days following inoculation were sacrificed and subjected to the same postmortem examinations.

---

[7] Kolmer, J. A., Bondi, A., Jr., Warner, F. H., and Dietz, C., *Science*, 1946, **104**, 315.

[‡] Kindly supplied by the Abbott Laboratories, North Chicago, Ill.

TABLE II. Streptomycin, Diasone and Combined Streptomycin-Diasone Therapy in the Treatment of Experimental Tuberculosis of Guinea Pigs.

Treatment	No.	Duration of life (days after inoculation)	Results of autopsies* Spleen	Liver	Lungs
Streptomycin	1	Died 31 days	++	+	+++
	2	” ” ”	++	+	+++
	3	” 32 ”	+++	++	++
	4	Killed 50 ”	+++	+++	++
	5	” ” ”	+++	+	++
	6	” ” ”	+++	+	++
	7	Died 30 ”	++++	+++	+++
	8	” ” ”	++++	+++	++
Diasone	9	Killed 50 ”	—	+++	++
	10	” ” ”	+++	+++	+++
	11	Died 48 ”	++++	++++	+++
	12	Killed 50 ”	—	—	++
	13	” ” ”	++	+++	++
	14	Died 49 ”	+++	+++	++++
	15	Killed 50 ”	—	—	+++
	16	” ” ”	+	—	+++
Streptomycin and Diasone	17	Killed 50 days	++	+	++
	18	” ” ”	+++	+	++
	19	” ” ”	++	+	+++
	20	” ” ”	+++	—	++++
	21	” ” ”	++++	++	++
	22	” ” ”	++	—	++
	23	” ” ”	—	+	++
	24	” ” ”	—	+	+++
Controls	25	Died 43 ”	++++	++++	+++
	26	Killed 50 ”	++++	+++	+++
	27	Died 45 ”	++++	++++	++++
	28	” 38 ”	++++	++++	+++
	29	” 34 ”	++++	++++	+++
	30	” 29 ”	+++	+++	++++
	31	” 27 ”	+++	—	++++
	32	” 32 ”	+	++	++++

* — = no lesions; + = occasional small tubercles or one small solitary tubercle; ++ = many microscopic tubercles; +++ = moderate involvement showing focal rather than diffuse tuberculosis with or without necrosis; ++++ = severe progressive tuberculosis with necrosis.

The results are shown in Table II and may be summarized as follows: (a) 7 of the 8 untreated control animals succumbed to extensive tuberculous infection in 23 to 43 days after inoculation; (b) 5 of the 8 animals treated with streptomycin succumbed in 30 to 32 days after inoculation; (c) 2 of the 8 animals treated with diasone succumbed in 48 and 49 days respectively after inoculation and (d) all animals treated with diasone and streptomycin survived. It has been very difficult to evaluate the results in relation to the distribution and severity of tuberculous infections of the spleen, liver and lungs, but in general terms it appears that these were somewhat less severe among the animals treated with streptomycin and diasone alone and by streptomycin and diasone.

Discussion. Apparently the maximum tolerated dose of streptomycin for guinea pigs inoculated with virulent human tubercle bacilli is about 6000 units per day (approximately 10,000 to 15,000 units per kg) when administered subcutaneously or intramuscularly for 5 to 10 weeks in succession. Undoubtedly the compound has shown some degree of therapeutic effectiveness in the treatment of experimental tuberculosis of guinea pigs in confirmation of previous reports[1-3] as based upon the survival rate of treated animals and the results of macroscopic and microscopic examinations of the spleen, liver and lungs for tuberculous lesions in postmortem examinations. In this connection, however, it is to be emphasized that our method of treatment was not maximum since but 2 doses of 1500 units of streptomycin in saline solution were given 4 hours apart with a 3rd dose of 3000 units in peanut oil and 3% beeswax 4 hours later per day, especially since it appears that streptomycin in peanut oil and beeswax is not as readily absorbed from the muscles as penicillin. Under the circumstances diasone by ingestion has yielded somewhat better therapeutic results while combined treatment with streptomycin and diasone has shown better results than observed with either compound alone, suggesting synergistic or additive therapeutic effects.

Summary. Both streptomycin and diasone are therapeutically effective in the treatment of experimental tuberculosis of guinea pigs. Both are more effective in suppressing tuberculous infection by the institution of treatment within a few days after inoculation with virulent tubercle bacilli than in the treatment of established lesions developing 2 weeks or longer after infection has been established. Particularly noteworthy therapeutic results were observed following combined streptomycin and diasone therapy, suggesting that these 2 compounds exert a synergistic or additive therapeutic effect in the treatment of experimental tuberculosis.

15561

Acquired Resistance of *Treponema pallidum* to Penicillin.

John A. Kolmer and Anna M. Rule.

From the Research Institute of Cutaneous Medicine, Philadelphia, Pa.

Various pathogenic penicillin-susceptible bacteria have been found to apparently acquire resistance to penicillin both *in vitro* and *in vivo*. The evidence, however, is not conclusive since so-called acquired resistance may be nothing more than the survival of variants in cultures of any bacterium possessing a higher natural resistance than usual when exposed to the compound *in vitro* or *in vivo*.

Be that as it may, the question of whether or not *T. pallidum* may develop so-called acquired resistance or tolerance to the compound is one of great practical importance and especially in relation to the treatment of chronic syphilis. In this connection Tung and Frazier[1] have reported that cultures of the Reiter strain of alleged *T. pallidum* did not develop any evidences of increased resistance or tolerance to penicillin after being subjected to 15 passages through media containing the compound although Dunham and his associates[2] have reported that the inadequate treatment of a syphilitic rabbit resulted in acquired resistance of *T. pallidum* to penicillin *in vitro*.

In our experiments 4 rabbits (first series) were inoculated intratesticularly with the Nichols-Hough strain of *T. pallidum*. About 5 weeks later all showed acute syphilitic orchitis with strongly positive dark-field examinations. Two rabbits were then given 500 units and 2 1000 units of penicillin* by intramuscular injection in sterile saline solution at 9 a.m., 1 p.m. and 4 p.m. daily for 9 doses, totalling 4500 and 9000 units respectively per kg of weight, over a period of 3 days. All showed clinical improvement with negative dark-field examinations in from 7 to 14 days after the institution of treatment but all thereafter relapsed clinically with positive dark-field examinations.

At the end of 3 weeks 4 rabbits (second series) were inoculated intratesticularly with testicular material obtained from the testicles of the 4 treated animals of the first series. Acute syphilitic orchitis with strongly positive dark-field examinations developed in 5 to 6 weeks at which time the animals received the same treatment as described above. All showed marked clinical improvement with negative dark-field examinations about 7 days after the institution of treatment which persisted over a period of about 2 weeks when 3 of the treated animals relapsed clinically with positive dark-field examinations. One rabbit, however, receiving a total of 9000 units per kg remained clinically and dark-field negative over a period of 6 weeks but lymph node transfers to fresh animals at that time gave positive results, indicative of incomplete "cure."

---

[1] Tung, T., and Frazier, C. N., *Am. J. Syph., Gon. and Ven. Dis.*, 1946, **30**, 205.

[2] Dunham, W. B., Hambre, D. M., McKee, C. M., and Rake, G., Proc. Soc. Exp. Biol. and Med., 1944, **55**, 158.

* Commercial penicillin (lot No. 45072102) kindly supplied by the Commercial Solvents Corp., New York, N.Y.

At the end of 3 weeks 4 rabbits (third series) were inoculated intratesticularly with testicular material obtained from 3 of the animals of the second series. All developed acute syphilitic orchitis with strongly positive dark-field examinations about 5 weeks later at which time they received the same treatment as previously employed. All animals showed healing with negative dark-field examinations about 7 days after the institution of treatment. Three rabbits, however, relapsed clinically with positive dark-field examinations about 5 weeks after the institution of treatment; the fourth animal receiving a total of 9000 units per kg remained clinically well, with negative dark-field examinations, up to 6 weeks at which time lymph node transfers were made with positive results.

At the end of 3 weeks 4 rabbits (fourth series) were inoculated intratesticularly with testicular material obtained from 3 of the animals of the third series. All developed acute syphilitic orchitis with strongly positive dark-field examinations about 5 weeks later at which time they received the same treatment as previously employed. All animals showed healing with negative dark-field examinations about 6 days after the institution of treatment. Two animals receiving a total of 4500 units per kg relapsed clinically with positive dark-field examinations about 4 weeks after the institution of treatment. The 2 animals receiving a total of 9000 units per kg, however, remained clinically well with negative dark-field examinations and at the end of 8 weeks lymph node transfers were made; one was positive and the second negative over a period of 9 weeks, indicative of a complete cure.

At the end of 3 weeks 8 rabbits (fifth series) were inoculated intratesticularly with testicular material obtained from the 2 rabbits of the fourth series with relapsing infections. Acute syphilitic orchitis developed about 5 weeks later with strongly positive dark-field examinations at which time treatment was instituted. Two rabbits were given intramuscular injections of 100 units per kg at 9 a.m., 1 p.m. and 4 p.m. for 10 doses (totalling 1000 units); both showed clinical improvement with negative dark-field examinations but relapsed at the end of about 10 days. Two rabbits were given a total of 5000 units per kg with temporary negative dark-field examinations but likewise relapsed. Two rabbits were given a total of 10,000 units per kg; both became dark-field negative in about 72 hours which persisted over a period of 6 weeks. At the end of that time lymph node transfers were made and the animals kept under observation for 3 months; one was positive and the second negative, indicative of complete biologic cure. Two rabbits were given a total of 50,000 units per kg; both became dark-field negative in about 72 hours which persisted over a period of 8 weeks. At the end of that time lymph node transfers were made and the animals kept under observation for 3 months; both were negative and indicative of complete biologic cure.

At the same time 8 additional rabbits were inoculated intratesticularly with the original or parent strain of *T. pallidum* as controls. Acute testicular syphilis developed in all in about 5 weeks with strongly positive dark-field examinations at which time treatment was instituted. The latter was exactly as described above, 2 rabbits receiving a total of 1000 units, 2 a total of 5000 units, 2 a total of 10,000 units and 2 a total of 50,000 units per kg. Practically identical results were observed.

Summary. The minimal curative dose of a commercial penicillin in aqueous solution by intramuscular injection 3 times a day for 10 doses in succession was apparently slightly more than 1000 units per kg (totalling 10,000 units) and probably somewhat less than 5000 units per dose (totalling 50,000 units) in the treatment of acute testicular syphilis of rabbits inoculated with the Nichols-Hough strain of *T. pallidum*. This strain did not show any evidence of acquired resistance or tolerance to penicillin after 3 consecutive passages through the testicles of rabbits treated with subcurative amounts of the compound.

15562

Streptomycin in Treatment of Acute Syphilitic Orchitis of Rabbits.

John A. Kolmer, Anna M. Rule, and Albert J. Paul.
From the Research Institute of Cutaneous Medicine, Philadelphia, Pa.

Dunham and Rake[1] have reported that the smallest curative dose of streptomycin in the treatment of experimental syphilis of rabbits varied from 187,000 to 374,000 units per kg of weight. In their experiments rabbits were inoculated intracutaneously with the Nichols strain of *T. pallidum* and treatment started within 3 days thereafter. The compound was administered intravenously every 4 hours over a period of 4 days. Since treatment was started before lesions had developed the results were more in relation to the prophylactic than the curative activity of streptomycin in this experimental infection. In parallel experiments the smallest effective dose of crystalline penicillin G was about 147 units per kg of weight so that this compound was about 3000 times more effective than streptomycin.

In our experiments 8 rabbits were inoculated intratesticularly with the Nichols-Hough strain of *T. pallidum*. Treatment of 6 animals was instituted about 6 weeks later at which time acute syphilitic orchitis had developed with strongly positive dark-field examinations; 2 rabbits were kept as untreated controls. Streptomycin* dissolved in sterile saline solution was injected intramuscularly at 10 a.m., 1 p.m. and 4 p.m. daily over a period of 8 days (24 injections). Clinical records and dark-field examinations were made every 2 days over a period of 10 weeks. Two animals were given 1000 units per kg, totalling 24,000 units; 2 were given 5000 units per kg, totalling 120,000 units and 2 were given 10,000 units per kg, totalling 240,000 units.

Serum assays were conducted according to the method of Stebbins and Robinson[2] using *Staphylococcus aureus* (SM strain). One-half to one hour after the 1000 unit dose per kg the serum levels showed from 3.0 to 4.1 units, 3.0 to 16.0 units after the 5000 unit dose per kg and 5.5 to 23.0 units after the 10,000 unit dose per kg per cc of serum.

At the end of the period of treatment one rabbit receiving a total of 24,000 units per kg showed some clinical improvement with negative dark-field examinations; similar results were observed with both rabbits receiving a total of 240,000 units per kg. Two weeks after treatment, however, all 3 of these animals showed relapsing infections with positive dark-field examinations. None of the remaining 3 treated animals showed the slightest clinical improvement and were persistently dark-field positive throughout the period of 10 weeks. Under the circumstances lymph node transfers to fresh animals were not made.

Summary. Streptomycin by intermittent intramuscular injection was slightly but temporarily effective in the treatment of 3 out of 6 rabbits with established acute syphilitic orchitis in total dosage of 24,000 to 240,000 units per kg of weight, but complete cures were not observed in any of the animals receiving total dosages of 24,000, 120,000 and 240,000 units per kg by intramuscular injection, over a period of 8 days.

---

[1] Dunham, W. B., and Rake, G., *Science*, 1946, **103**, 365.

* Streptomycin sulfate kindly supplied by the Abbott Laboratories, North Chicago, Ill.

[2] Stebbins, R. B., and Robinson, H. J., Proc. Soc. Exp. Biol. and Med., 1945, **59**, 255.

15563

Radiopotassium Autography of the Rat's Brain.

HARRY F. COLFER AND HIRAM E. ESSEX.

From the Division of Experimental Medicine, Mayo Foundation, Rochester, Minn.

The autoradiographic technic provides an interesting and valuable approach to the histophysiology of certain radioactive isotopes in studies in detail of their intratissue distribution. Thus far the method has been applied chiefly to studies of radioactive iodine metabolism in the thyroid.[1-3] Radioiodine is concentrated in the thyroid to a much greater extent than in any other tissue,[1] whereas K^{42} is taken up very slowly and in small amount by the brain.[4] For this reason the feasibility of K^{42} autography of the brain might be questioned. Actually, however, the procedure does not offer any unusual difficulties.

The sequence of operations was as follows: weighing of the rats; intraperitoneal injection of a dose of K^{42} proportional to the weight of the animal; anesthetization, after a period allowed for assimilation of the K^{42}, and removal of the brain by craniotomy; fixation of brain slices 3 to 4 mm thick in absolute alcohol; infiltration of the tissues with 50% xylene and 50% paraffin, and then with paraffin alone; blocking, sectioning and fixing the microtome sections to glass microscope slides; exposure of a small strip of X-ray film to each slide; and development of the exposed film.

The K^{42}, as the chloride, was injected intraperitoneally in a 5.44% aqueous solution, the dose being 0.75 millicuries per 100 g of body weight. Following this, a period of 12 to 18 hours was allowed for the radioactive potassium to approach equilibrium in the brain, since this substance has been shown by Noonan, Fenn and Haege[4] to have a very slow turnover in this organ.

In this connection it was necessary also to consider the rather short half life of K^{42} (12.4 hours). It was found that if longer periods were allowed after injection, the decay of the isotope more than offset the advantage of a closer approach to equilibrium.

The dose employed was relatively large because it has been shown that only a very small amount of injected K^{42} is taken up by the brain, as compared with other tissues,[4] and because a certain minimum of radioactivity within the tissue sections is necessary to bring about adequate exposure of even the most sensitive photographic emulsions, especially when very long exposure times are precluded by a short half life of the isotope.

The fixative used was absolute alcohol, because it is necessary to exclude any water, which would affect distribution of electrolytes within the tissue. This brought about sufficient hardening of the tissue slices within 2 hours. An hour in warm xylene-paraffin, followed by an hour in paraffin alone, brought about satisfactory infiltration.

The optimal thickness for the microtome sections was found to be 6 to 12 μ. Thicker or thinner sections did not cut well. Furthermore very thin sections did not contain enough of the radioactive potassium to produce an adequate exposure of the photographic emulsion.

The ribbons of tissue sections were fixed to the microscope slides simply by slight warming to cause the paraffin of the sections to fuse to the glass. The operation also helped to bring about flattening of the sections on the slides. It was found that the autoradiographs were less distinct if the tissue sections were cleared of paraffin with xylene and were fixed to the slides by dipping in thinned collodion.

Alternate stained sections were prepared from each tissue specimen, to serve as histologic controls for the autoradiographs.

---

[1] Hamilton, J. G., Soley, M. K., and Eichorn, E. B., *Univ. Calif. Publ., Pharmacol.*, 1941, **1**, 339.

[2] Gorbman, Aubrey, and Evans, H. M., *Endocrinology*, 1943, **32**, 113.

[3] Leblond, C. P., Fertman, M. B., Puppel, I. D., and Curtis, G. M., *Arch. Path.*, 1946, **41**, 510.

[4] Noonan, T. R., Fenn, W. O., and Haege, Lorraine, *Am. J. Physiol.*, 1941, **132**, 474.

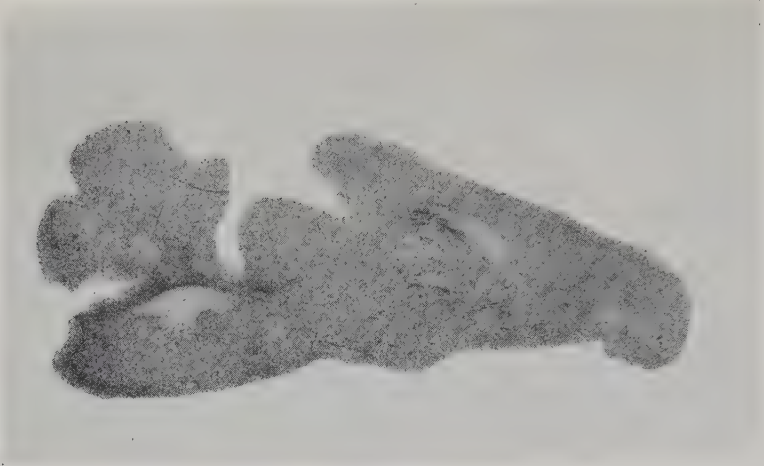


Fig. 1.
Autoradiograph showing distribution of K^{42} in a sagittal section of the brain of a normal rat 8 μ thick. The cerebral area of the autoradiograph is at the right and the cerebellar and brain-stem area at the left (× 5).

Some preparations also were made by microincinerating (according to the method of Scott[5]) sections which contained K^{42}. It was thought that removal in this way of the organic component of the tissue sections might eliminate a certain barrier between the radioactive substance and the photographic film but there was no improvement in the quality of the autoradiographs obtained by this method.

Dental X-ray films were used in the form of strips cut at a slightly greater width than that of the tissue sections. Three such strips could usually be obtained from each film. A strip of film and a tissue preparation were bound directly together, in the dark room, by wrapping them in black paper. It was important to weigh down each such film-slide preparation during the exposure period to insure intimate contact between the film emulsion and the tissue sections.

The optimal exposure time was found to range between 12 and 24 hours, depending on the thickness of the sections. If a few extra preparations are made for each experiment, films can be developed at intervals to determine when the exposure has been adequate for that particular experiment.

In a series of 16 experiments highly satisfactory autoradiographs of rats' brains were obtained by this means (Fig. 1). The normal distribution of K^{42} was found to be fairly homogeneous throughout the brain, though there frequently was the indication, by relatively greater areas of density in the autoradiographs, of somewhat greater concentrations in the cerebral and cerebellar cortex, the basal ganglia or the roof of the fourth ventricle.

Summary. The critical points of technic for the preparation of radiopotassium autographs of rats' brains have been discussed and it has been shown that good results can be obtained despite the fact that this isotope has a short half life and enters brain tissue very slowly.

It was found by this means that the distribution of K^{42} in the brains of normal rats is fairly homogeneous except for some tendency to greater concentration in the cerebral and cerebellar cortex, the basal ganglia and the roof of the fourth ventricle.

---

[5] Scott, G. H., *Protoplasma*, 1933, **20**, 133.

15564

Experimental Burns Accompanied by Histamine Administration Abets the Ulcer Diathesis.*

Stanley R. Friesen and Owen H. Wangensteen.
From the Department of Surgery, University of Minnesota, Minneapolis, Minn.

The spontaneous occurrence of ulceration in the duodenum or stomach of man following burns has been recognized for more than 100 years. In an extensive review of the literature, Harkins[1] noted among 680 patients dying of burns submitted to necropsy 26 ulcers, an incidence of 3.8%, with a ratio of duodenal to gastric ulcer of 4.5 to 1.

The explanation of the occurrence of ulcer following burns is as yet unknown. The pathological changes which occur most commonly in the gastrointestinal tract following burns consist of hyperemia and congestion.[2,3] Necheles and Olson[4] studied gastric secretion in dogs with isolated gastrostomized stomachs following torch burns to the hind leg of the animal. They noted that such burns augmented gastric secretion, if infusions of fluid were given at the time of study. Hartman[5] was unable to confirm these findings, and found that both the free hydrochloric acid and the total acid were reduced following burns. Hartman, employing live steam and a 50 to 60% burn in dogs in a period of observation extending up to 13 days, observed ulceration in the duodenum and stomach in 63.3% of the animals. In another series in which tanning agents were employed as a lenitive to infection, the incidence of spontaneous ulcer was 6.6%.

It is the purpose of this study to determine whether experimental burns accelerate the occurrence of the histamine-in-beeswax provoked ulcer. In rabbits bona fide ulcers cannot be produced with histamine alone.[6] In dogs, Hay and his associates[6] noted that 40 days were necessary to produce ulcer with regularity in all dogs injected. In Hay's series of 12 dogs, 23 days was the average time for ulcer production employing the histamine-in-beeswax technic. In another series of 14 control dogs to which 30 mg of histamine-in-beeswax was administered daily, killed from 4 to 14 days after commencement of administration of histamine, ulcer was observed in one dog, after 7 daily doses of histamine. Over a 6-year interval during which time histamine-in-beeswax has been given to a large number of dogs, ulcer has been observed twice at 4 days following the daily administration of 30 mg of histamine implanted in beeswax.

Methods. These experiments were carried out on dogs and rabbits in 3 series. The method of burn used in each series consisted of immersion in water at 100°C, the body surface area and period of exposure varying in the 3 series. Immersion was found to be a satisfactory method of producing burns in that accurate standardization is possible, the tissue is not broken at the time of burn, and no added factor of pressure is present. All animals were anesthetized with sodium pentobarbital, 15 mg per pound body weight intravenously. In almost all instances, the anesthesia was adequate to obviate pain or any suggestion of discomfort on the part of the animal during the burning process; occasionally, Demerol, 50 mg IM was used. The hair was closely clipped prior to scalding. No postoperative treatment of the burn

---

* The researches upon which this presentation is based were supported by the Augustus L. Searle Fund for Experimental Surgical Research, the Citizens' Aid Society, the Dr. and Mrs. Harry B. Zimmermann Fund for Experimental Surgical Research, and by grants of the Graduate School of the University of Minnesota.

[1] Harkins, Henry N., *Surgery*, 1938, **3**, 608.

[2] Pack, George T., *Arch. of Path. and Lab. Med.*, 1926, **1**, 767.

[3] Harkins, Henry N., *Arch. Path.*, 1944, **38**, 147.

[4] Necheles, H., and Olson, Wm. H., *Surgery*, 1942, **11**, 751.

[5] Hartman, F. W., *Ann. Surg.*, 1945, **121**, 54.

[6] Hay, L. J., Varco, R. L., Code, C. F., and Wangensteen, O. H., *Surg., Gyn., and Obst.*, 1942, **75**, 170.

TABLE I.
Dogs Subjected to 40% Burn Accompanied by Administration of Histamine-in-Beeswax Mixture.

Dog No.	Wt in lb	Daily dose, histamine base mg	No. of injections	Results	Remarks
6	20	30	1	Petechial bleeding Gastric ulcers	Sacrificed 24 hr, melena
7	22	30	1	Bleeding gastric ulcer	Died 2nd day, exsanguination
8	40	30	1	Bleeding duodenal ulcers; hemorrhagic gastritis	Died within 24 hr; exsanguination
9	33	30	1	Bleeding duodenal ulcers	Same
10	30	30	1	” ” ”	”
24	32	30	5	Duodenal ulcers; one perforated	Died 5th day; peritonitis
25	22	30	3	Pyloric and duodenal ulcers	Died 3rd day
31	30	30	1	Two duodenal ulcers	Sacrificed in 24 hr
Controls (40% burn but no histamine)					
5	20	0	0	Antral erosion	Sacrificed in 24 hr
11	37	0	0	No ulcers or erosions	Same
26	26	0	0	Minute bleeding; ulcers	Died on 6th day
27	31	0	0	Marked congestion; no ulcers	” ” 2nd ”
28	15	0	0	No ulcers or erosions	” ” 4th ”
29	24	0	0	” ” ” ”	” ” 5th ”
30	30	0	0	” ” ” ”	” ” 5th ”
Controls					
1	33	30	2	No ulcers or erosions	Sacrificed after 2 inj.
2	24	30	3	Same	” ” 3 ”
3	20	30	4	”	” ” 4 ”
4	38	30	5	”	” ” 5 ”

TABLE II.
Dogs Subjected to 15% Burn Accompanied by Administration of Histamine-in-Beeswax.

Dog No.	Wt in lb	Daily dose, histamine base mg	No. of injections	Results	Remarks
12	34	30	2	Antral erosions	Died 2nd day
13	22	30	5	Bleeding duodenal ulcers	Died exsanguination 5th day
17	23	30	5	Bleeding duodenal ulcers; one perforated	Died 5th day, peritonitis
18	33	30	2	Bleeding pyloric ulcers	” 2nd day
19	32	30	5	Pyloric ulcers	” 5th ”
22	27	30	1	Bleeding antral erosion	” 1st ”
Controls (15% burn, but no histamine)					
14	29	0	0	No ulcers or erosions	Sacrificed 5th day
15	41	0	0	Bleeding duodenal ulcers; gastric petechiae	Died 9th day
20	25	0	0	Antral petechiae; no ulcers	” 14th ”
21	32	0	0	No ulcers or erosions	” 14th ”

was instituted. Histamine-in-beeswax mixture prepared after the method of Code and Varco[7] was injected intramuscularly (30 mg base in the dog and 15 mg base in the rabbit) each evening, the first injection being given 2 hours prior to the burn. The dogs' feed pans were removed and no more food given until the following morning. No restriction of food intake was exercised in the instance of the rabbits. The time of sacrifice in the dogs was determined by the occurrence of spontaneous hematemesis or

---

[7] Code, C. F., and Varco, R. L., *Am. J. Physiol.*, 1942, **137**, 225.

TABLE III.
Rabbits Subjected to 40% Burn Accompanied by Administration of Histamine-in-Beeswax Mixture.

Rabbit No.	Size	Daily dose of histamine base	No. of inj.	Results
1	Adult	15	1	Fundic ulcer, bleeding petechiae
5	”	”	2	Bleeding fundic ulcer with bleeding petechiae
6	”	”	2	(Same)
7	”	”	2	Few petechiae
8	”	”	2	Bleeding fundic ulcer
9	”	”	1	Numerous antral ulcers
11	”	”	2	Petechiae fundus and duodenum
12	”	”	2	Small bleeding antral ulcer
16	”	”	3	Few petechial bleeding points
Controls (40% burn but no histamine)				
2	”	0	0	No petechiae, erosion or ulcers
3	”	0	0	Bleeding petechiae fundus
4	”	0	0	(Same)
10	”	0	0	No petechiae, erosions or ulcers
13	”	0	0	” ” ” ” ”
14	”	0	0	(Same)
15	”	0	0	”

melena or extreme weakness. The rabbits were sacrificed at varying periods of time. In all animals sections of stomach, duodenum, the burned area, brain, liver, kidney, lung and adrenal were obtained for microscopic observations and further study.

Experiments. Series I. Fifteen dogs were used in this series. Each dog received a 40%, 3rd degree burn by immersion in 100°C water for 10 seconds. Eight of these dogs received daily injections of histamine-in-beeswax mixture.

Series II. Ten dogs were used in this series. Each dog received a 15%, 3rd degree burn by immersion in 100°C water for 20 seconds. Six of these dogs received daily injections of histamine-in-beeswax mixture.

Series III. Sixteen rabbits were used in this series. Each rabbit received a 40%, 3rd degree burn by immersion in 100°C water for 5 seconds. Nine of these rabbits received daily injections of histamine-in-beeswax mixture.

Results. Series I. (Table I) All of the 8 dogs receiving a 40%, 10-second burn accompanied by histamine-in-beeswax administration developed ulcers within 5 days. Six of the 8 dogs died of exsanguination or were sacrificed within 24 hours, all showing ulceration, 5 actively bleeding. One of the 7 dogs not receiving histamine-in-beeswax injections developed minute-gastric-bleeding ulcers in 6 days.

Series II. (Table II) All of the 6 dogs receiving a 15%, 20-second burn accompanied by histamine-in-beeswax administration showed erosion or ulcer within 5 days. Four of the 6 showed bleeding ulcers within 5 days. One of the 4 dogs not receiving histamine-in-beeswax injections showed bleeding duodenal ulcers with gastric petechiae at 9 days. Another with burn alone showed antral petechiae in 14 days; the other 2 dogs showed no significant gastrointestinal pathologic change, other than congestion, in 5 and 14 days.

Series III. (Table III) Seven of the 9 rabbits receiving a 40%, 5-second burn accompanied by histamine-in-beeswax administration showed erosion, ulcer, or petechial bleeding points within 2 days. Two of the 7 rabbits subjected to burn alone showed only petechial bleeding points of the fundus within 2 days.

Four control dogs receiving daily injections of histamine-in-beeswax mixture alone failed to develop ulceration when sacrificed in 2 to 5 days. All the animals subjected to burns displayed gastrointestinal hyperemia and congestion, especially of the duodenum and stomach at necropsy.

Discussion. It is apparent from these experiments that burns abet the ulcer diathesis; that is, ulcers are provoked more readily and quickly with histamine in the presence of gastrointestinal congestion resulting from experimental burns. The evidence of gastric and/or duodenal ulceration in animals subjected to experimental burns depends upon the type, degree, extent, therapy, and period of observation of the burn. The incidence of ulcers in untreated dogs subjected to burns alone in this study was 2 of 11 dogs, or 18.2%; 7 of the 11 dogs died or were sacrificed within 5 days; the 2 dogs developing ulcers died on the 6th and 9th day. It is possible that, if the survival time in these animals were prolonged, the incidence of spontaneous ulceration in dogs with burn alone would be greater. Of the 14 dogs subjected to burning in addition to histamine administration, all showed erosion or ulcer within 5 days. Twelve of the 14 presented definitive ulcers, 8 of which were actively bleeding.

Conclusions. 1. Experimental burns, when accompanied by administration of histamine-in-beeswax mixture, produce gastric and/or duodenal ulceration in which the incidence of hemorrhage is high. 2. Experimental burns abet the ulcer diathesis.

15565

Morphologic Effect of Desoxycorticosterone Acetate on the Thymus.*

P. Dontigny (Introduced by H. Selye.)

From the Institut de Médecine et de Chirurgie expérimentales, Université de Montréal, Montréal, Canada.

A reciprocal relationship between adrenal and thymus size has been discussed by several investigators.[1-6] Adrenal enlargement caused by nonspecific damage,[3] by adrenocorticotrophin[7-11] or treatment with corticoids[12-15] is accompanied by thymus atrophy. In the absence of the adrenals, however, neither damage nor adrenocorticotrophin produce this effect.[12]

Wells and Kendall[15] claimed that though several glucocorticoids, such as dehydro-corticosterone, corticosterone and Kendall' compound "E," produce this response, desoxy-corticosterone and its acetate (D.C.A.) d not at the doses of 10 mg every 3rd day for 9 days. This latter finding is in dis-agreement with that of Selye[16] and Sely

---

* This investigation was subsidized by a grant of the Commonwealth Fund. The author is also greatly indebted to Dr. E. Schwenk of the Schering Corporation, of Bloomfield, N.J., who supplied the D.C.A.

[1] Hammett, F. S., *J. Metabolic Res.*, 1925, **78**, 91.

[2] Anderson, D. H., *J. Physiol.*, 1935, **85**, 162.

[3] Selye, H., *Endocrinology*, 1937, **21**, 169.

[4] Dohan, F. C., Proc. Soc. Exp. Biol. and Med., 1942, **49**, 404.

[5] Ingle, D. J., Proc. Soc. Exp. Biol. and Med., 1938, **38**, 443; 1940, **44**, 174.

[6] Kendall, E. C., *Ann. Rev. Biochem.*, 1941, **10**, 285.

[7] Cramer, W., and Horning, E. S., *Lancet*, 1939, **1**, 192.

[8] Moon, H. D., Proc. Soc. Exp. Biol. and Med 1940, **43**, 42.

[9] Noble, R. L., and Collip, J. B., *Endocrinology* 1941, **29**, 234.

[10] Houssay, B. A., *J. Am. Med. Assn.*, 194 **118**, 833.

[11] Dougherty, T. F., and White, A., Proc. Soc Exp. Biol. and Med., 1943, **53**, 132.

[12] Selye, H., *Brit. J. Exp. Path.*, 1936, **17**, 23

[13] Carriere, G., Morel, J., and Ginesti, P. J *C. R. Biol. Paris*, 1937, **126**, 46.

[14] Ingle, D. J., *Endocrinology*, 1939, **24**, 194.

[15] Wells, B. B., and Kendall, E. C., *Proc. Sta Meet. Mayo Clinic*, 1940, **15**, 133.

[16] Selye, H., *Canad. Med. Assn. J.*, 1940, **42**, 11

[17] Selye, H., and Albert, S., *Am. J. Med. Sc* 1942, **204**, 876.

TABLE I.
Effect of D.C.A. on Thymus, Iliac Lymph Nodes, Uterus and Body Weight.

Group	No. of animals	Body wt, g		Thymus wt, mg	Iliac lymph nodes wt, mg	Uterus wt, mg
		Initial	Final			
Cortical extract	9	76	75	198 ± 10.3*	13 ± 0.5	111 ± 8.9
D.C.A.	8	74	76	132 ± 7.5	13 ± 1	97 ± 7.8
Control	7	73	70	251 ± 14.3	12 ± 0.7	36 ± 3.4

* Means and standard errors.

TABLE II.
Effect of D.C.A. on Thymus, Iliac Lymph Nodes, Uterus, and Body Weight.

Group	No. of animals	Body wt, g		Thymus wt, mg	Iliac lymph nodes wt, mg	Uterus wt, mg
		Initial	Final			
Control extract	11	82	95	146 ± 9.8	15 ± 1	68 ± 4.7
D.C.A.	7	85	98	80 ± 5.8	19 ± 1.5	89 ± 12.7
Control	8	84	86	220 ± 16	18 ± 1.2	55 ± 5.4
Absolute control	10	81	99	179 ± 15	15 ± 0.9	53 ± 8.2

and Albert[17] who obtained marked thymic atrophy in normal male rats when sufficiently high doses of D.C.A. (10 mg daily for 14 days) were administered.

It is possible that in the experiments of Selye the high doses were damaging and so indirectly produced thymic atrophy through the animal's own adrenals. In order to clarify this problem an investigation has been performed concerning the effect of D.C.A. on the thymus of adrenalectomized rats, in which nonspecific damage could not be operative.

Experimental. Two series of experiments were performed. In the first, 30 female albino rats weighing 65 to 80 g were employed. All were adrenalectomized and maintained on 1% NaCl instead of drinking water. Treatment was begun 24 hours postoperatively. The animals were divided into 3 equal groups. The first group received 1 cc of cortical extract (Connaught Laboratories, Toronto) divided into 2 daily subcutaneous injections. The second group was injected with 10 mg of D.C.A. suspended in 0.4 cc of water in 2 daily subcutaneous injections. The third group received 10 mg of cholesterol in the same manner as the D.C.A. group and served as controls. During the first 2 days of treatment a few animals, which did not appear to be entirely healthy, were sacrificed. At the end of 5 days treatment all remaining animals were killed with chloroform and bled. The thymus, iliac lymph nodes and uterus were fixed in "Suza" then dissected and weighed. The results are summarized in Table I which indicates that while the thymus weights in the animals of the 2 treated groups were markedly reduced, no change was seen in the weights of the lymph nodes. The uterus on the other hand was stimulated both by cortin and by D.C.A.

In order to confirm these observations a second experiment was performed. The procedure was the same as that employed above, except that the duration of treatment was 6 instead of 5 days and all doses were doubled. The additional D.C.A. and cholesterol were administered intraperitoneally. A fourth intact control group was also added.

Table II summarizes the results of this experiment and indicates that here again, in the corticoid-treated groups, the thymus and the uterus are influenced in the same manner as they were in the first series.

Microscopically the structure of the thymus was characterized by a marked diminution of thymocytes with some proliferation of the stroma in both corticoid-treated groups, especially in that receiving D.C.A. In the same groups the uterus and vagina revealed histologic signs of stimulation, similar to that obtained by a mixture of folliculoid and luteoid substances.

Discussion. The above experiments show that short-term treatment with large doses of D.C.A. causes thymus atrophy. The fact that entirely negative results have been obtained by other investigators[15,18,19] can probably be explained by the relatively small doses of D.C.A. employed. On the other hand the length of the treatment is also important since Selye[20] has shown that continued treatment with D.C.A. not only fails to cause atrophy but increases the size of the thymus.

It must be remembered that thymus atrophy is elicited not only by corticoids. Selye[21] has shown that in chronically-treated rats all steroid hormones cause a certain degree of atrophy in this gland and that all hormonally active steroids have some folliculoid activity. Furthermore this author[22] claimed that in adrenalectomized animals the only hormones which are able to cause thymus involution are the corticoids and the folliculoids.

It has not been proven yet, whether or not the antithymic action of D.C.A. is due to its folliculoid property. This possibility should not be neglected. In this regard it will be interesting to determine whether folliculoids influence the thymus and the lymphatic organs in the same manner as do the glucocorticoids, namely whether they cause lymphocyte breakdown and release of gamma globulins which are not influenced by D.C.A.[23,24] in the doses employed.

Summary. In short-term experiments, large doses of desoxycorticosterone acetate cause marked thymus atrophy. Thus it appears that this effect is not the exclusive characteristic of the glucocorticoids and furthermore that the nonspecific damage of D.C.A. has not been operative since the result was obtained in adrenalectomized rats.

---

[18] White, A., and Dougherty, T. F., PROC. SOC. EXP. BIOL. AND MED., 1944, **56**, 26.

[19] Carnes, W. H., Ragan, C., Ferrebee, J. W., and O'Neil, J., *Endocrinology*, 1941, **29**, 144.

[20] Selye, H., *J. Anat.*, 1941, **76**, 94.

[21] Selye, H., *Rev. Canad. Biol.*, 1942, **1**, 577.

[22] Selye, H., Harlow, C., and Collip, J. B., *Endokrinologie*, 1936, **18**, 81.

[23] Dougherty, T. F., and White, A., *Endocrinology*, 1944, **35**, 1.

[24] White, A., and Dougherty, T. F., *Endocrinology*, 1945, **36**, 207.

15566

Prolongation of Consciousness in Anoxia of High Altitude by Glucose.*

AUSTIN H. RIESEN, T. N. TAHMISIAN, AND COSMO G. MACKENZIE.

From the Yerkes Laboratories of Primate Biology and Yale University; the Department of Medicine, the University of Chicago; and the Department of Biochemistry, Cornell University Medical College.

Dietary factors relating to anoxia tolerance in the human have been put to test recently in the investigations of King *et al.*,[1] Green *et al.*,[2] and Eckman *et al.*[3] These workers have been concerned with the effects of diet on visual, psychomotor, and mental performance in persons exposed to intermediate degrees of oxygen deprivation such as occur at altitudes of 15,000 to 17,000 feet. The comparisons made show the contrasting effects of meals varying with respect to carbohydrate, protein, and fat content. High carbohydrate diets were found to increase significantly performance at these comparatively low altitudes.

---

* The views expressed in this paper do not necessarily reflect those of the Army Air Forces. The experimental work was done in the Altitude Training Section at Drew Field, Fla.

[1] King, C. G., Bickerman, H. A., Bouvet, W., Harrer, C. J., Oyler, J. R., and Seitz, C. P., *J. Av. Med.*, 1945, **16**, 69; King, C. G., Bouvet, W., Crook, M. N., Harrer, C. J., Oyler, J. R., and Schwimmer, D., *Science*, 1945, **102**, 36.

[2] Green, D. M., Butts, J. S., and Mulholland, H. F., *J. Av. Med.*, 1945, **16**, 311.

[3] Eckman, M., Barach, B., Fox, C. A., Rumsey, C. C., and Barach, A. L., *J. Av. Med.*, 1945, **16**, 328.

The work herein reported was started prior to our knowledge of the studies cited above. While dealing with the same general problem, it represents a different approach, for 3 reasons. First, the carbohydrate and/or vitamin C are here added to a diet of choice. Second, the data are obtained at higher altitudes (27,000 and 30,000 ft.) where fulminating anoxia rapidly leads to unconsciousness and death. Third, in our experiments the time between disconnection of the oxygen mask and unconsciousness serves as the measure of the effect of the supplementary feedings. From the standpoint of their application to the practical problems of high altitude flight, these data are highly pertinent. In critical situations such as bail out, loss of cabin pressure, or failure of oxygen systems additional seconds of consciousness can mean the difference between life and death.

Methods. Details of the testing procedure with samples of handwriting criteria are given in a previous publication devoted to the establishment of an altitude versus time curve for the duration of consciousness.[4] The same endpoint for measuring the duration of "useful consciousness" was employed in the present investigation. Thus, inability to continue writing served as a definable index of a person's degree of helplessness. Whereas in some cases recollection of events and the self-realization of inability to perform were reported afterwards by our subjects, even here there was an undeniable condition of dependence upon the intervention of another person to prevent complete insensibility.

Subjects used in the main series of tests were members of B-17 combat crews in training at Drew Field. The interval between their previous meal, which was any meal of the day, and the time of supplemental feeding varied from ½ to 3 hours, averaging approximately 2 hours. In one separately conducted series of tests nonflying personnel of the Medical Detachment served in experimental and control groups. These men ingested the solutions approximately one hour after breakfast.

The fliers were tested during their crew's regularly scheduled training flight in the altitude chamber. They were given only the information that volunteers were needed for an experiment on the effect of vitamins and sugar on a flier's ability to withstand oxygen lack. It was desired that 4 of their number drink the apparently identical solutions, and that during the course of the subsequent chamber flight they disconnect their masks at high altitude, and write dictated dittys as long as possible. Assurance was of course given that they would be reconnected whenever they showed inability to continue writing.

From among the volunteers 4 individuals drew lots to determine which one of the differently numbered solutions each was to drink. The contents of the solutions were as follows:

1. 0.2 g saccharine and 1.0 g ascorbic acid.
2. 0.2 g saccharine and 0.1 g citric acid.
3. 80.0 g dextrose (anhydrous) and 1.0 g ascorbic acid.
4. 80.0 g dextrose (anhydrous) and 0.1 g citric acid.

The volume of each solution was 350 cc. Citric acid served as the control ingredient for the vitamin C. Saccharine sweetened the solutions not containing dextrose. Comments made by the subjects about the taste of the solutions showed that they were unable to discern any differences. No subject entered into the experimental results more than once.

The order of testing the subjects at altitude was randomized so as to eliminate any constant difference in time interval before testing the effect of a particular solution and to control differences in mental attitudes of subjects who had or had not observed anoxic performances of fellow crew members before it was their turn. In the experiments conducted at 27,000 ft. the first of the 4 anoxic

---

[4] Mackenzie, C. G., Riesen, A. H., Bailey, J. R., Tahmisian, T. N., and Crocker, P. L., *J. Av. Med.*, 1945, **16**, 156.

TABLE I.

Prolongation by Glucose of the Duration of Consciousness in Trained Fliers Exposed to Anoxia at 27,000 ft. in the Altitude Chamber. Tests Were Conducted 30 to 50 Minutes Following the Oral Administration of Supplements. Quantities for the Solutions Are Given in the Text.

	Solutions			
	1 Saccharine, ascorbic acid	2 Saccharine, citric acid	3 Glucose, ascorbic acid	4 Glucose, citric acid
No. of cases	19	20	19	19*
Mean duration of consciousness (sec.)	192.1 ± 12.9	182.9 ± 10.8	252.3 ± 17.7	269.6 ± 25.8
S.D.	54.3	47.7	74.2	108.4

	Solutions	
	1 & 2 combined (no glucose)	3 & 4 combined (glucose)
No. of cases	39	38*
Mean duration of consciousness (sec.)	187.4 ± 8.2	260.9 ± 15.1
S.D.	51.3	93.3

* One additional case lasting over 900 sec. omitted from calculations.

episodes was begun either half an hour or one hour after the ingestion of the solutions. All 4 subjects were then tested within a period of 20 minutes. On the 30,000 ft. flights only the shorter (half hour) interval was used.

Results. At 27,000 ft. the effects of preflight ingestion of carbohydrate and/or vitamin C on our measure of the duration of consciousness 30 to 50 minutes later are shown in Table I. Mean durations with their standard errors are given in seconds. Comparing the effect of solution No. 1 with No. 2 and that of No. 3 with No. 4 we find no significant differences between the means of each pair, the t-values being 0.5 and 0.6, respectively. This absence of any measurable effect of the vitamin C was consistent in all groups tested.

Comparing group No. 3 with its saccharine-fed control group, No. 1, gives a value of t of 2.8, and a comparison of No. 4 and No. 2 a value of 3.2, either of which taken separately indicates significance at the 1% level. A critical ratio of 4.3 results if No. 1 and No. 2 are combined for a comparison with No. 3 and No. 4. The difference of 73.5 seconds between the means of the combined groups represents an advantage of 39% in the duration of consciousness for the men receiving glucose over those in the control groups.

TABLE II.

Effect of Glucose on Mean Duration of Consciousness of Air Crew Members Exposed to Anoxia at 30,000 ft. in the Altitude Chamber, and Tested 30 to 50 Minutes After Ingestion of the Solutions.

	Solutions	
	1 & 2 (no glucose)	3 & 4 (glucose, 80 g)
No. of cases	14	16
Mean duration of consciousness (sec.)	126.0 ± 7.2	147.6 ± 6.0
S.D.	25.7	23.1

At 30,000 ft. (Table II) the increase associated with glucose ingestion is less both in absolute terms and relative to the performance of the controls, the increase being 17%. The small numbers of cases render these figures less reliable than in the tests at 27,000 ft., and contribute to the lesser degree of significance found in the difference: $t = 2.3$, significant at the 5% level.

The results when a longer time interval followed drinking of the solutions are given in Table III. As compared with the men tested 30 to 50 minutes after treatment (Table I), a much smaller increase in the duration of consciousness is shown by the air crew members with 60 to 80 minutes elapsing between the ingestion of glucose and the tests at 27,000 ft. The difference at this longer interval in the experienced subjects

TABLE III.
Effect of Glucose on Mean Duration of Consciousness of Trained Fliers and of Nonflying Personnel Exposed to Anoxia at 27,000 ft. in the Altitude Chamber. All Tests Were Conducted 60 to 80 Minutes Following the Oral Administration of Supplements.

Subjects		Solutions 1 & 2 (no glucose)	 3 & 4 (glucose, 80 g)
Air crew members	No. of cases	26	21
	Mean duration of consciousness (sec.)	201.9 ± 10.3	223.8 ± 17.4
	S.D.	51.7	78.0
Nonflying personnel	No. of cases	17	18
	Mean duration of consciousness (sec.)	170.8 ± 10.8	218.0 ± 12.2
	S.D.	43.3	50.9

does not meet the statistical criterion for significance.

Also shown in Table III are data with men inexperienced in flight, either actual or as simulated in the altitude chamber. When no glucose was administered these groups became incapacitated in a somewhat shorter time than corresponding groups made up of air crew members. However, the increase in the duration of consciousness produced by glucose feedings proves to be significant at the 1% level, $t = 2.9$. The lesser degree of variability in this group is presumably at least in part a reflection of the greater constancy of the time elapsing after the preceding meal. The carbohydrate had an effect which apparently counteracted factors that were operating in this group to reduce its average period of performance, so that after glucose the inexperienced subjects did as well or better than did the fliers in the control group.

Discussion. Our results show that the duration of consciousness at high altitudes following interruption of the oxygen supply is extended by the previous administration of glucose. This is compatible with the increase in performance observed by King *et al.*[1] and Green *et al.*[2] at lower altitudes following high carbohydrate meals.

The more marked increase in duration of consciousness at 27,000 ft. as compared with 30,000 ft. was to be expected from our earlier findings[4] on the relation between the duration of consciousness and altitude. At 26,000 ft. lowering the actual altitude by 2,000 ft. resulted in a greater prolongation of consciousness than decreasing any higher altitude by the same amount.

The greater tolerance to anoxia of the *untreated* fliers as contrasted with the *untreated* nonflying personnel is in harmony with our previous demonstration that men who have received training in the reactions to anoxia, as had the fliers, become unconscious less rapidly than do untrained individuals. Presumably this is due to a minimizing of the excitement and fear engendered by contemplating and experiencing the sudden loss of supplementary oxygen, and to the greater confidence in and familiarity with the oxygen mask and related equipment. Fear and excitement are believed to be potent factors in reducing tolerance to anoxia.

King *et al.*[1] and Green *et al.*[2] are of the opinion that high carbohydrate meals increased the performance of individuals in their tests at 15,000 to 17,000 ft. because of the higher carbon dioxide yield together with a minimum metabolic need for oxygen. The parallel physiological and psychological measurements of Eckman *et al.*[3] have furnished experimental confirmation of this hypothesis. The improvement in performance reported by King and associates, however, was somewhat greater than that found or accounted for by Eckman *et al.*

While our own conditions are not strictly comparable to the work cited above either with respect to the method of administering carbohydrate or with respect to the type of anoxia studied, it is reasonable to suppose that the beneficial effect obtained with glucose at high altitude was likewise associated with an increase in the R.Q. The temporal relationships also offer some evidence on the mechanism involved. Protection against anoxia in the experienced fliers was greater

30 to 50 minutes after glucose ingestion than it was after 60 to 80 minutes. The former time corresponds closely with the occurrence of the maximum level of glucose in the blood in glucose tolerance tests on normal individuals. Unfortunately it was impossible for us to measure blood glucose or R.Q.

Conclusions. 1. The preflight administration of a single dose of glucose in water to individuals on a normal diet produced a significant increase in the resistance to unconsciousness from anoxia at 27,000 and 30,000 ft. 2. The protection afforded flying personnel by the glucose solution was greater 30 to 50 minutes following its administration than after an interval of 60 to 80 minutes. 3. At 27,000 ft. the mean duration of consciousness was increased by approximately 40%, or more than one minute, by the preflight ingestion of glucose solution. 4. Vitamin C, either alone or in conjunction with glucose, had no demonstrable effect on the duration of consciousness.

15567

Total Peptic Activity of Gastric Juice. A Method of Determination.*

HARRY H. LEVEEN. (Introduced by Dallas B. Phemister.)

From the Department of Surgery, The University of Chicago.

It is the purpose of this paper to assess critically the present status of tests for the determination of pepsin and to describe an improved method.

Since different proteins differ in their rate of digestibility, it is necessary to choose either a highly purified protein for use as a substrate, or at least a substance of constant composition so that results be reproducible with different lots of substrate. Partial denaturization of a protein by coagulation renders it a mixture of different proteins. Because one can rarely be sure denaturization is complete, tests using coagulated protein substrates[1-3] are subject to error. Gelatin has been employed as a substrate protein,[4,5] but it is not ideal since it hydrolyzes in acids without pepsin.[6] One test uses fresh milk as a substrate.[7] Although the composition of "Klim," dried milk powder, is quite constant, individual samples of fresh milk are not constant.[8]

Pepsin is unlike many inorganic catalysts whose presence in traces is sufficient to bring about a change. With pepsin, the rate of conversion is directly proportional to the concentration of active enzyme and active substrate; *i.e.*, the reaction follows the law of mass action. However, the reaction rate is not uniform for more than a brief period (Fig. 1) because peptones formed during digestion combine with the enzyme to form an inactive pepsin-complex.[9] Pepsin + Peptone $\rightleftarrows$ pepsin-peptone. It is the concentration of peptone that determines how much pepsin is active. When the reaction rate has leveled off, the addition of a sizable increment of pepsin will but slightly increase the rate of digestion because of the high concentration of peptones already present. Dilution by decreasing the concentration of the inhibitor allows digestion to continue.[9] The

---

* Done on a grant from the Otho S. A. Sprague Memorial Institute.

[1] Mett—Hawk and Bergein, *Practical Physiological Chemistry*, 11th Ed., 1937, p. 308.

[2] Neilson, C. H., and Bonnot, E., *Arch. Int. Med.*, 1913, **11**, 395.

[3] Riggs, B. C., and Stadie, W. C., *J. Biol. Chem.*, 1943, **150**, 463.

[4] Osterberg, A. E., Vanzant, F. R., and Alvarez, W. C., *J. Clin. Invest.*, 1933, **12**, 551.

[5] Gilman, A., and Cowgill, G. R., *J. Biol. Chem.*, 1930, **88**, 743.

[6] Northrop, J. H., *J. Gen. Physiol.*, 1921, **3**, 715.

[7] Barowsky, H., Tauber, H., and Kleiner, I. S., *Am. J. Digest. Dis.*, 1937, **4**, 229.

[8] Northrop, J. H., *J. Gen. Physiol.*, 1932, **16**, 41.

[9] Northrop, J. H., *J. Gen. Physiol.*, 1920, **2**, 471.

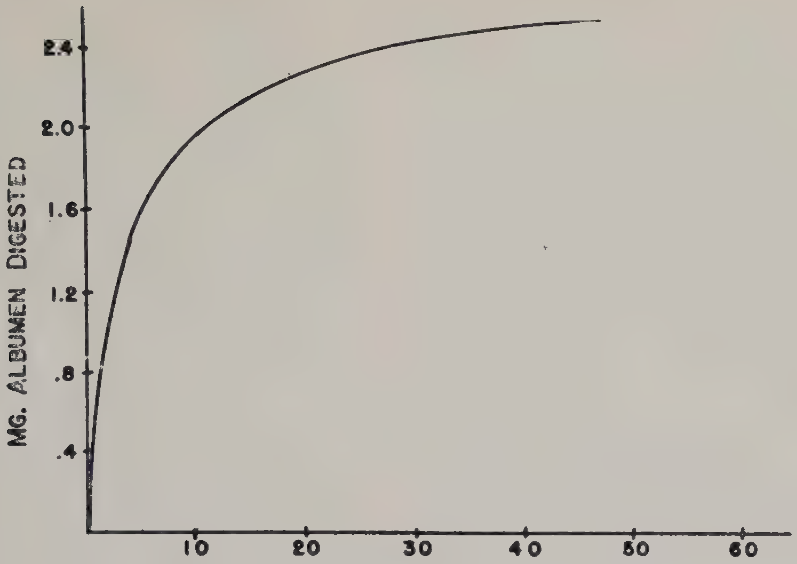


FIG. 1.
Milligrams of protein digested plotted against time.

pepsin of gastric juice is in combination with inhibitory substances which are probably peptones. It is rapidly and completely freed from the inhibitors by dilution. Some authors have found the separation usually to be complete at a 1/100 dilution.[10] It follows then that total pepsin can only be assayed on highly diluted gastric juice, since in low dilutions one is assaying not total pepsin but a varying mixture of pepsin and pepsin-inhibitor complex. One should therefore avoid tests using fixed low dilutions[2,3,11–13] and varying dilutions as an integral part of the test.[7,14]

A linear relationship does not exist between pepsin concentration and quantity of protein digested.[9] One should be able to express results in amount of protein digested (since it is a measure of peptic power) or in some figure proportional to the pepsin concentration. The liberation of tyrosine during digestion of a hemoglobin substrate occurs almost in direct proportion to enzyme concentration.[15] In the test to be described, the mg albumen digested are proportional to the logarithm of the pepsin concentration. In most tests, the kinetics of the reaction have not been investigated.

Although most methods for determining pepsin fail to meet all the criticisms, one modification of Anson and Mirsky's method is satisfactory.[10] This new modification, as stated by the authors and by the experience of others, requires considerable technical skill and is somewhat time-consuming. These factors render its use of limited value in most clinical and investigative work. For this reason, the method of Mett is still extensively used although its extremely wide range of error has been known for some time.[16]

Previous tests have one great disadvantage: there is no scheme for assaying total pepsin and the percentage of pepsin inhibited in gastric juice.

Dennis and Ayer[17] have described an accurate method for the determination of pro-

---

[10] Bucher, G. R., Grossman, M. I., and Ivy, A. C., *Gastroenterology*, 1945, **5**, 501.

[11] Waldschmidt-Leitz, E., and Kustner, G., *Physiol. Chem.*, 1927, **171**, 70.

[12] Bucher, G. R., and Beazell, J. M., *Am. J. Physiol.*, 1941, **133**, 230.

[13] Rose, W. C., *Arch. Int. Med.*, 1913, **5**, 459.

[14] Hackett, W. R., *Lancet*, 1943, **1**, 430.

[15] Anson, M. L., and Mirsky, A. E., *J. Gen. Physiol.*, 1932, **16**, 59.

[16] Cobb, P., *Am. J. Physiol.*, 1905, **13**, 448.

[17] Ayer, J. B., Dailey, M. E., and Fremont-Smith, F., *Arch. Neurol. and Psych.*, 1931, **26**, 1038.

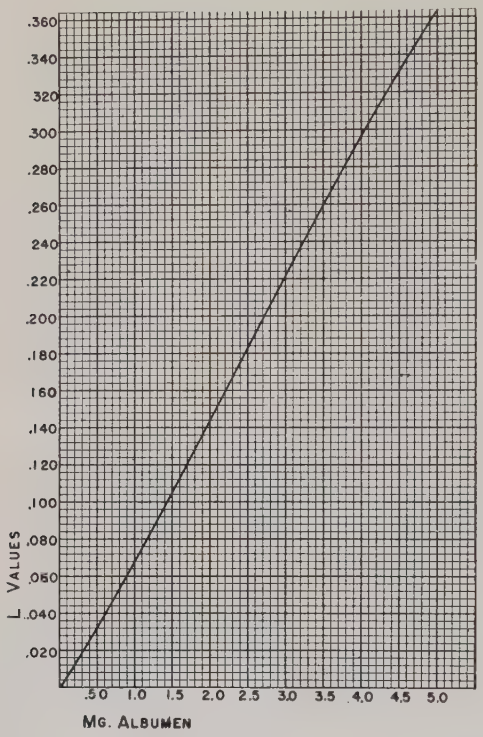


FIG. 2.
The optical density of sulfosalicylic acid precipitated egg albumen, expressed as "L" values, is plotted against the concentration of egg albumen.

tein in cerebrospinal fluid using sulfosalicylic acid as a precipitant and measuring the resultant turbidity quantitatively in a colorimeter. This method can be adapted to the determination of peptic power utilizing albumen as a substrate.

Preparation of Albumen Substrate. "Impalpably powdered, dried egg albumen" (Merck) is dissolved in distilled water so that each cc contains 1 mg. Before diluting to the mark, a few cc of 1/1000 aqueous merthiolate is added as a preservative. A drop of caprylic alcohol may be added to prevent foaming. The solution is placed in a refrigerator for 2 days and then filtered and refiltered until crystal clear (rarely centrifugation is necessary).

The amount of protein nitrogen in solution is only 70% as determined by the Kjeldahl nitrogen method. Since the units are arbitrary, the results are calculated from the weight of the dried powder.

Dried egg albumen is prepared from many sources of eggs and its composition is quite uniform. A small portion of the albumen is denatured in processing but remains insoluble. Nephelometric readings on various fresh lots of dried albumen have yielded almost identical results. In addition, their digestibility varied but slightly. It is believed that this protein is a satisfactory substrate for practical use. (A more sensitive alternate method using crystalline protein will also be discussed).

Solutions prepared as described are stable for many months if kept in a refrigerator. If a slight turbulence develops, the solution can be decanted or filtered from time to time. It is best to change the solutions once a month.

Standardization of Albumen Solution. Density curves are made under the conditions obtained in the performance of the test. The albumen solution is diluted so that 5 cc will contain 0 to 5 mg of albumen.

Five cc of these dilutions are placed in Evelyn photoelectric colorimeter tubes and 4 cc of N/20 HCl is added to each. One cc of .5 N NaOH is added. The total volume is then brought to 24 cc by the addition of 14 cc of 6% sulfosalicylic acid. (The quantities are for direct reading with an Evelyn photoelectric colorimeter: other colorimeters may accommodate but part of the total mixture). After inverting 3 times *(do not shake)* the tubes are read in the colorimeter using a No. 420 filter. If the "L values" for the galvanometer readings are plotted against albumen concentrations, a line is obtained which is almost straight (Fig. 2).

The particle size varies with the concentration of albumen. The readings are a mixture of transmitted light (specular density) and light reflected from particles nearest the photoelectric cell (diffuse density). For these reasons, Beer and Lambert's law does not hold and a straight line relationship between optical density (L value) and concentration of albumen is not to be expected. The practical range over which reproducible nephelometric readings are possible is 0 to 5 mg of egg albumen.

Choice of Dilution for Performance of Test. Bucher *et al.*[10] have indicated that above a 1/100 dilution, augmentation of pepsin ac-

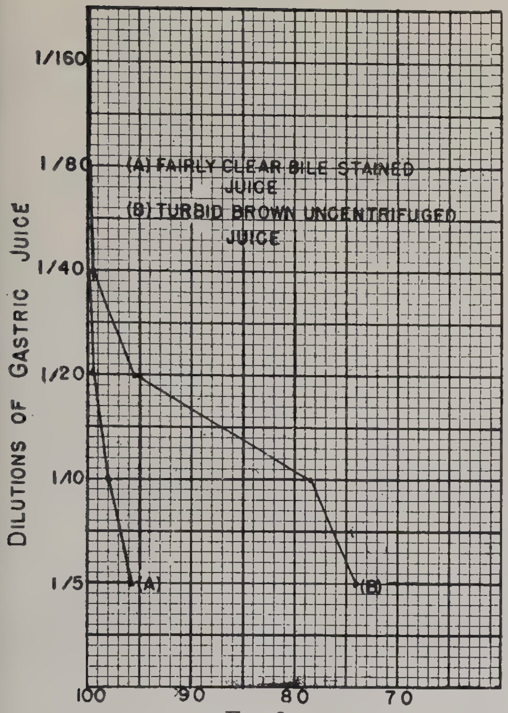


Fig. 3.
The optical density (galvanometer readings only) of gastric juice precipitated with sulfosalicylic acid is plotted. One hundred represents the light transmission of distilled water.

tivity does not occur with further dilution. In rare exceptions, a dilution of 1/200 is necessary. For this reason the actual final dilution in the performance of the test is 1/563.

Choice of Digestion Time. Digestion does not proceed at a uniform rate for more than a brief period (Fig. 1). This interval is a matter of seconds in the described test, rendering accurate measurement most difficult. A digestion time of 30 minutes allows for the greatest and most uniform change.

Choice of Substrate Concentration. A substrate concentration of 5 mg permits the assay of wide variations in pepsin concentration and yet remains within a reasonable range of accuracy for the nephelometric method. Rarely, specimens of canine gastric juice reach the upper limit of the range, but none have necessitated change in method.

Digestion pH. The digestion pH repeatedly determined by the glass electrode is 1.75.

Procedure for Performance of Test. To 5 cc of albumen solution is added 4 cc of 1/250 dilution of gastric juice in N/20 HCl. (The author pipettes 0.2 ml of gastric juice into 50 cc N/20 HCl). The tubes are placed in a constant temperature water bath at 37°C for ½ hour. Digestion is then terminated by the addition of 1 cc of N/2 NaOH. Incubation is continued for ½ hour following which 14 cc of sulfosalicylic acid are added. The alkali and additional period of incubation are necessary for complete inactivation of pepsin, which does not occur with sulfosalicylic acid alone. The sulfosalicylic acid precipitate may otherwise be digested. Densimetric readings are made 15 minutes after the addition of sulfosalicylic acid.

Calculation of Result. The number of milligrams of albumen remaining undigested is determined from the colorimeter reading by referring to the standardization curve. (Fig. 3). This amount subtracted from 5 mg, the amount initially present, yields the number of milligrams of albumen digested by 2/125 cc of gastric juice. The result may be left in this form (peptic power) or converted into pepsin units.

If the mg albumen digested by a known quantity of crystalline pepsin† is plotted on semilog paper, a straight line is obtained; the quantity of albumen digested being proportional to the logarithm of the pepsin concentration. Expressed in another way, the concentration of crystalline pepsin is proportional to the antilog of the quantity albumen digested. The results are merely expressed in the latter proportionality units (designated crystalline pepsin equivalents) because the action of various crystalline pepsins differs.[18]

Illustrative Example. Crystalline pepsin equivalents are obtained by finding the antilogarithm of the milligrams albumen digested using the digits to the right of the decimal point as the logarithm and those to the left as the mantissa.

1. 1.25 mg albumen digested

---

† Crystalline pepsin was obtained through the kindness of Dr. R. M. Herriot of Rockefeller Research Foundation and by purchase from Plaut Research Laboratories, Bloomfield, N.J.

[18] Wiss, O., *Helvetica Chimica Acta,* 1946, 29, 237.

Antilog .25 with a mantissa of 1 = 18

2. 3.76 mg albumen digested

Antilog .76 with a mantissa of 3 = 580

Since the figure obtained is for 2/125 of a cc of gastric juice, the pepsin equivalents must be multiplied by 62.5 to obtain pepsin units per cc. To complete the example:

1. Pepsin unit of 18 × 62.5 = 1125
2. Pepsin unit of 580 × 62.5 = 36,250

Reproducibility. Twenty-five separate determinations of mg albumen digested were made using single samples of artificial canine and human gastric juice. The mean deviation was in the neighborhood of 2% in each case. The greatest deviation in any one sample was 5%.

Sources of Error. The density of the gastric juice (which contains protein itself) was determined in various dilutions by precipitation with 6% sulfosalicylic acid. (The same quantity of gastric was used as in the performance of the test except that distilled water was substituted for albumen solution). With uncentrifuged turbid gastric juice, there is practically no optical density attributable to gastric juice alone at a dilution of 1/250 (Fig. 3). In rare cases, where the gastric juice is alkaline, a sufficient quantity of mucus may be in solution to influence the densimetric readings. In such cases it is a good practice to do a blank determination as described above and subtract the optical density due to mucus. Some gastric juice may be bile colored—at 1/250 there is no evidence of color. If the juice is very highly colored a blank may be prepared from the diluted juice by substituting distilled water for albumen.

Sulfosalicylic acid occasionally takes on a slight brown color. In this case, a water blank should not be used but a blank containing 10 cc of water and 14 cc of 6% sulfosalicylic acid.

Failure to obtain good mixture at any stage of the procedure can produce a large error. Failure to invert the tubes several times before reading can produce a considerable error.

If greater accuracy is desired in assaying juices of extremely low peptic activity, half concentrated substrate should be used. This dilution puts the nephelometric determination in its most sensitive range.

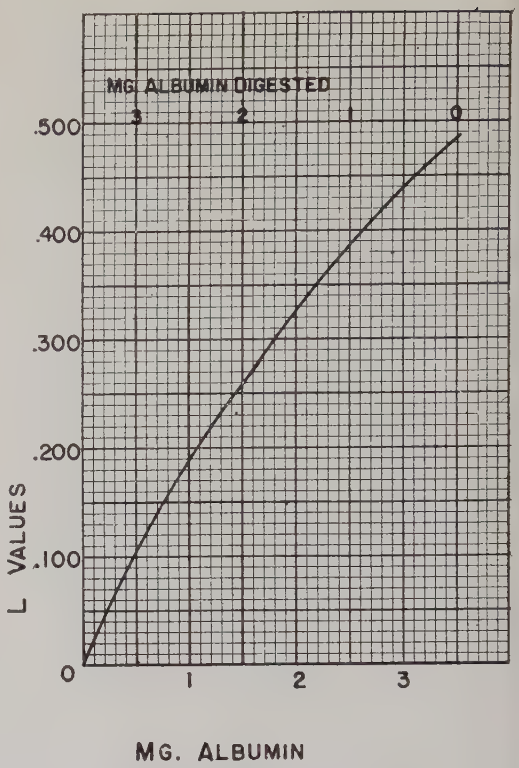


FIG. 4.

The optical density of sulfosalicylic acid precipitated albumin, expressed as "L" values, is plotted against the concentration of human serum albumin.

Alternative Method Using Purified Bovine or Human Albumin.‡ The nephelometric curve for human and bovine albumin is shown in Fig. 4. (The curves for human and bovine albumin are almost identical and may be so considered for practical purposes). The workable range for nephelometric determinations is from 0 to 3.5 mg under the experimental conditions given.

Human or bovine albumin is dissolved in distilled water so that each cc contains 2.1 mg.

One-tenth cc of gastric juice is diluted to 50 cc with N/20 hydrochloric acid. Five cc of diluent is mixed with 5 cc of albumin solution. Digestion is performed for 20 minutes at 37° C, at the end of which time the pepsin is inactivated by the addition of 2 cc of .4 N NaOH and incubation for 30 minutes at 37°C. Thereafter, 4 cc of the inactive mixture is pipetted into a colorimeter tube and 20 cc of 6% sulfosalicylic acid is added. After 15 minutes the optical density is read in the photoelectric colorimeter with a No. 420 filter.

---

‡ The highly purified human albumin was obtained through the courtesy of Dr. E. J. Cohn of the Harvard Medical School Serum Laboratory. The highly purified bovine albumin was obtained through the courtesy of Armour and Co. The fact that these materials will probably be available for general use in the near future makes their use ideal.

Since an aliquot of ⅓ is taken for the albumin determination, the mg albumin digested as obtained from Fig. 4 are multiplied by 3 to obtain the actual milligrams of pepsin digested.

When the mg albumin digested is plotted against the concentration of crystalline pepsin on semilogarithmic paper, a straight line is obtained. The range of this test is from 5 to 2000 γ. The antilogs of 0 to 3.5 would cover this range without distortion so that the antilog of the mg albumin digested unmultiplied by 3 is used to convert to pepsin equivalents.

It will be noted that the range of this procedure is much smaller than that utilizing egg albumen. Hence it may be occasionally necessary to use a greater initial dilution than is indicated. Although its range is smaller its sensitivity is correspondingly greater.

The digestion pH for this test has been found to be 1.75 as determined by the glass electrode.

To assay juice of extremely low peptic power it is more accurate if half concentrated albumin is used for the substrate.

Summary and Conclusion. The disadvantages and inaccuracies of many tests for pepsin have been discussed. A method for determining total pepsin which does not embody these disadvantages is described. Egg albumen is used as substrate and a photoelectric colorimeter used as a densimeter is employed to determine the amount of albumen solution digested. An alternative method of greater sensitivity is also described utilizing purified bovine and human serum albumin. Little technical skill is required and the execution of the tests is exceedingly rapid.

Thanks are expressed to Dr. William H. Fishman for his aid in the preparation of this manuscript.

15568

Active Pepsin Fraction, Resting Peptic Activity, and Percentage of Pepsin Inhibition in Gastric Juice.*

HARRY H. LEVEEN. (Introduced by Dallas B. Phemister.)

From the Department of Surgery, University of Chicago.

Total pepsin is only an index of the secretory activity of the pepsinogenic cells and gives no indication of the digestive action *in vivo* as modified by inhibitory substances and pH. Because of the importance of pepsin in experimental ulcer formation[1,2] one should know the quantitive effects of both inhibitory substance and pH.

Total pepsin can be assayed only after high dilution of gastric juice, whereupon it is completely freed from inhibitory peptones. If gastric juice were assayed in its undiluted state, only that portion of pepsin uncombined with inhibitor (active pepsin fraction) would be measured.

If digestion is carried out at different pH's, a correction chart for varying digestion pH's can be constructed. By this means, the actual digestive power of the juice can be obtained after ascertaining the pH of the gastric juice.

The digestive action of juice corrected for pH and inhibitor substances has been designated resting peptic activity, and is a measure of the digestive power which the intestinal mucosa is resisting.

---

* Done on a grant from the Otho S. A. Sprague Memorial Institute.

[1] Schiffrin, M. J., and Warren, A. A., *Am. J. Dig. Dis.*, 1942, **9**, 205.

[2] Driver, R. L., Chappell, R. H., and Carmichael, E. B., *Am. J. Dig. Dis.*, 1945, **12**, 166.

Preparation of Albumen Substrate. One hundred grams of dried egg albumen is dissolved in 300 cc of distilled water with frequent agitation over a period of hours. Merthiolate is added as a preservative. The insoluble material is cleared by filtering for 24 hours or by centrifugation.

Technic for Performance of Test. Three cc of albumen solution, ½ cc of .25 N HCl and 1½ cc of gastric juice, in order indicated, are pipetted into a test tube. After shaking, the mixture is incubated for 30 minutes at 37°C. Pepsin is inactivated at the end of the digestion period by the addition of 1 cc of N/2 NaOH, and, after shaking, incubation for 30 minutes. Three cc of the mixture is then diluted to 50 cc with N/150 NaOH. Five cc of this dilution is pipetted into an Evelyn colorimeter tube and 19 cc of 6% sulfosalicylic acid is added. After 15 minutes, the density is determined in the Evelyn photoelectric colorimeter. Because only 5 mg of the original 100 mg of albumen are taken for determination, the density curve previously obtained may be used. The mg albumen digested are obtained by subtracting from 5 mg, the possible total. Since an aliquot of 1/20 was taken for determination the result is multiplied by 20.

pH of Digestion Mixture. It is well nigh impossible to obtain adequate buffering by the usual methods; therefore, the pH of the digestion mixture is taken in each determination with a glass electrode. The mixture for pH determination is prepared as for digestion with the exception that boiled gastric juice is used (active digestion disturbs the pH readings).

Effect of pH upon Digestion. If digestion is performed at different pH's, a curve is obtained relating pH to the percentage of digestion at pH 1.75 which is chosen as 100% (Fig. 1). By means of this graph all results are corrected to pH 1.75 which is the digestion pH of the test for total pepsin.

Effect of Low Dilution on Digestion and Interpolation to Undiluted State. If the square root of the milligrams albumen digested per cc of gastric juice (after correction to pH 1.75) is plotted against dilution, a linear relationship is obtained (Fig. 2). This relationship holds up to a dilution of 1:5 in resting juices 8 to 12 hours old, but only up to 1:4 or 1:4.5 in the fresh juice of active secretion. It becomes feasible, therefore, to extrapolate to the point of no dilution without the introduction of a sizable error.

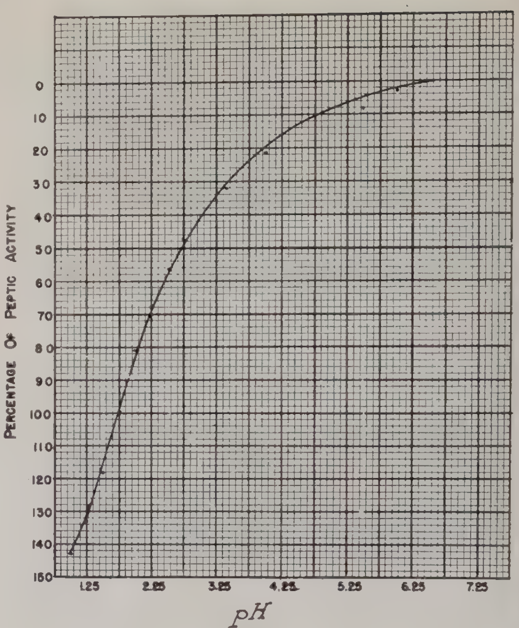


FIG. 1.

The percentage of egg albumen digestion as compared to pH 1.75 (which has been taken as 100%) is represented on the ordinate. The pH of the digestion mixture is represented on the abscissa.

The relationship presented is such that an increase of one in dilution increases the square root of the mg albumen digested by one. To extrapolate to point of no dilution from 3.33 dilution, subtract 2.33.

Calculation of Peptic Power. The actual calculation becomes as follows: mg albumen digested as obtained from the chart is multiplied by 20 and corrected to pH 1.75 using Fig. 1 and then divided by 1.5 (number of cc used for test). From square root of the result, 2.33 is subtracted. This final figure squared represents the number of mg albumen digested per cc of undiluted gastric juice at standard digestion pH of 1.75. To obtain the "power" of the juice at its own pH, the latter figure is adjusted to that pH by use of Fig. 1.

Conversion to Pepsin Units. The results

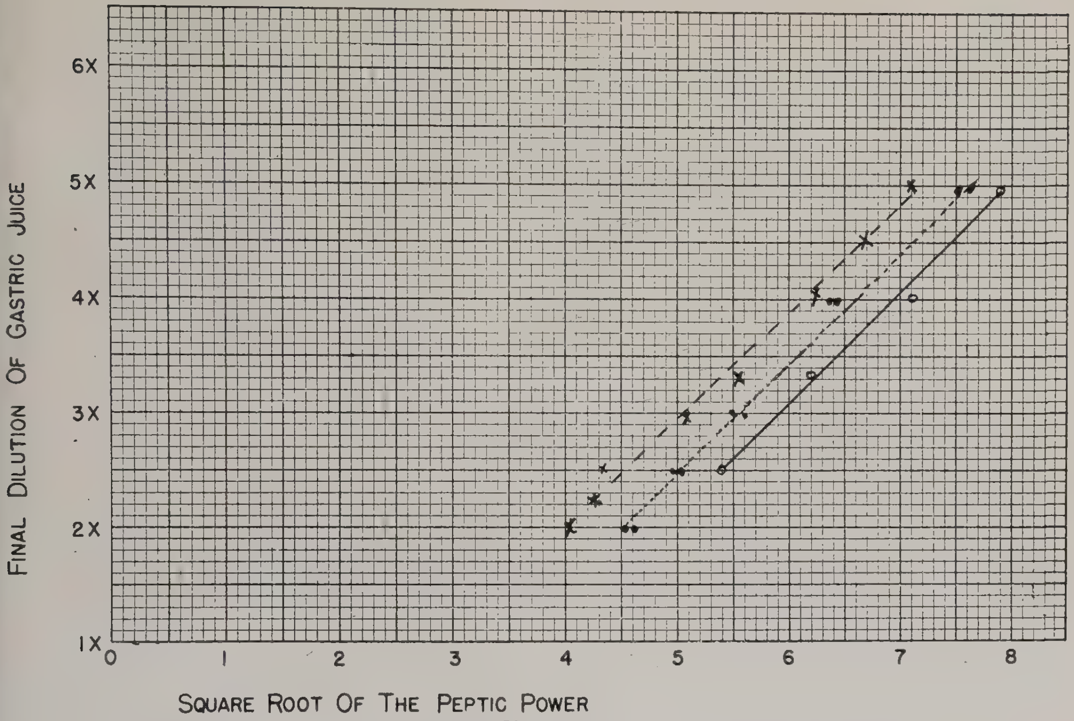


FIG. 2.

The square root of the milligrams of egg albumen digested per cc of gastric juice is plotted against dilution. A linear relationship is observed only in low dilutions (*i.e.*, not over 1 to 5). Interpolation to zero is done by simply extending the straight line to 1x which represents the undiluted state. The relationship is such that on all 3 different specimens plotted, an increase in dilution of 1 increases the square root of the milligrams albumen digested by one.

may be expressed in pepsin units so adjusted that the tests for total pepsin and active pepsin fraction are comparable. One can, thereby, determine that percentage of the total pepsin which is normally inhibited.

Determinations made with crystalline pepsin in both tests show that 2.5 times the enzyme concentration in the test using concentrated substrate produce 20 times the amount of digestion (Fig. 3). Hence, if the mg albumen digested in the concentrated test is divided by 20 and the antilogarithm of this result divided by 2.5, pepsin units for the active fraction and resting peptic activity are obtained which are comparable with the test for total pepsin.

It should be understood that conversion of the resting peptic power to pepsin units is an artificial comparison and that really the action of pH is on the substrate and not on the enzyme. It represents a comparable activity which would be obtained if the enzyme concentration were reduced and the pH remained constant.

Percentage of Pepsin Inhibited. Subtracting the active fraction from the total pepsin content (both being in identical pepsin units) yields that fraction of pepsin which is inactive. This latter figure divided by the total pepsin concentration and multiplied by 100 yields the percentage of pepsin inhibited.

Sources of Error. The sources of error are the same as in the test for total pepsin, with the exception that dissolved mucin in alkaline gastric juice can produce more major differences in nephelometric readings. This necessitates an occasional blank determination which is performed by pipetting .05 cc of gastric juice into 5 cc of distilled water and adding 19 cc of sulfosalicylic acid.

The sulfosalicylic acid must always be added to the albumen solution (not vice versa), and preferably at the same relative rate; otherwise large errors in the nephelometric readings may occur.

Alternative Method Using Crystalline Protein. Crystalline bovine or human albumin are used the same as described in the test for total pepsin.

Two cc of a 70 mg/cc solution of albumin is mixed with 1 cc of .15 N HCl and 2 cc of gastric juice. Incubation is performed for 20 minutes at 37°C after which inactivation is accomplished by the addition of 3 cc of .4 N NaOH and incubation for 30 minutes. Two cc is then diluted to 50 cc with N/150 NaOH. To 5 cc of the diluent are added 19 cc of sulfosalicylic acid. Since the range of this test is small, the digestion pH should be taken in advance and adjusted by altering the normality of the acid added to the digestion mixture so as to be in the range of pH 3.5. If the digestion is too great or too small, the digestion pH may be adjusted so as to bring the juice into the range of the test. Correction for pH is made according to Fig. 4.

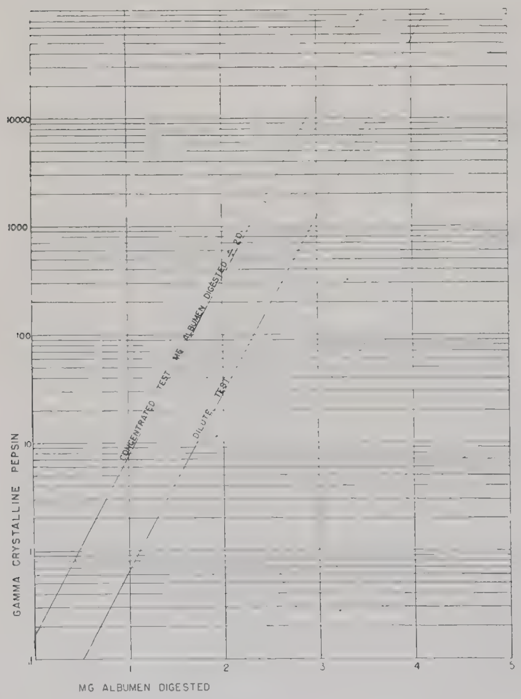


FIG. 3.
The relationship of the test using dilute albumen to that using concentrated albumen is shown. The plots are on semilogarithmic paper. Two and one-half times the crystalline pepsin concentration produces 20 times the amount of digestion in the test using concentrated albumen. The milligrams albumen digested in this latter test have been divided by 20.

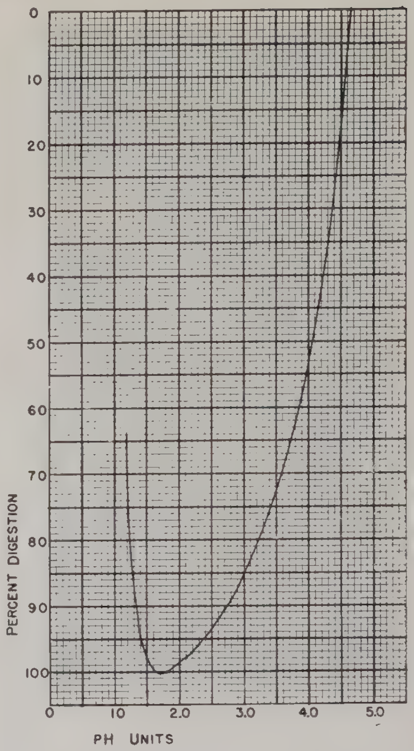


FIG. 4.
Shows relationship of pH of digestion mixture to percentage of digestion of human serum albumen at pH 1.75 which is represented as 100%.

In extrapolating to the undiluted state, the milligrams albumen digested, after pH correction, is divided by 2 (number of cc of gastric juice) and the square root taken. From this figure 1.03 is subtracted. This difference is squared and multiplied by 40, since an aliquot of 1/40 was taken for albumen determination. The result yields the milligrams albumen digested per cc of undiluted gastric juice. If the results are to be expressed in pepsin units, the multiplication by 40 is omitted.

If no correction is made for the aliquot taken for determination in the concentrated test, 13.5 times the amount of crystalline enzyme in the concentrated test digested an amount identical in the dilute test. Therefore, the antilog of the mg albumin digested (after correction for dilution and pH and unmultiplied by 40) is divided by 13.5. Pepsin units thus obtained are of the same magnitude as in the dilute test so that the percentage of pepsin inhibition may be obtained. Resting peptic power cannot be determined with the alternative method.

Summary and Conclusions. Simple procedures have been described whereby the amount of active pepsin, the percentage of pepsin inhibition and the resting peptic power can be obtained when used in combination with the test for total pepsin. The test employs a nephelometric method to determine the diminution of an albumen substrate after digestion.

15569 P

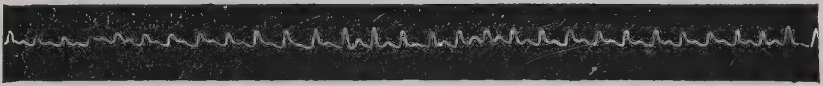
An Electro-Magnetic Sphygmograph[*] of New and Simple Design.

FRANCIS MARSH BALDWIN AND BASIL I. PANZER.

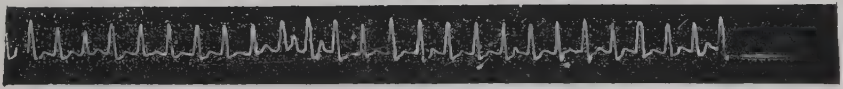
From the Physiological Laboratory and the Department of Physics, The University of Southern California.

While there are several more or less satisfactory mechanical devices adapted for the transcription of pulse pressure waves from superficial arteries (carotid,[†] radial,[‡] digital[§]), excellent results are not always easily attained. The adaptation of the electro-magnetic principle here described, while simple, seems to present a sturdy as well as a foolproof device. The chief assemblage includes a 200 ohm, single Button Model W carbon granule microphone[¶] of excellent sensitivity which, by the adaptation of a short rod fixed upon its diaphragm, is held or strapped over a suitable throbbing artery serving in place of a dispatching tambour. It is to be noted here that its function is the reception *not* of sounds but of the pulse pressure waves as such, and it acts as a variable resistance to the passage of an electric current. Thus the resistance is caused to vary inversely with the differential pressure throbs of the artery. At the recording end of the electrical circuit in this assemblage, a permanent cylindrical alnico magnet, ⅜″ in diameter and 5″ long is fixed in an upright position, supported upon an adjustable iron stand. About the magnet is placed an ordinary 6″ test tube with bottom removed, upon which is wound about 200 turns of 28 gauge cotton covered magnet wire of approximately 1.7 ohm total resistance; each end of

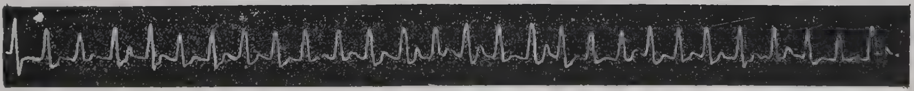
Normal radial



Slow closure of carotid



From femoral



---

[*] Acknowledgment is here made to Dr. R. E. Vollrath of the Physics Department for his interest and helpful suggestions.

[†] Usual assemblage of two Tambours in series.

[‡] The Dudgeon Sphygmograph.

[§] See A Simplified Digital Sphygmograph, Baldwin, F. M., *Science*, N. S. **69**, No. 1792, May, 1929.

[¶] Manufactured by Universal Microphone Co., Inglewood, Calif.

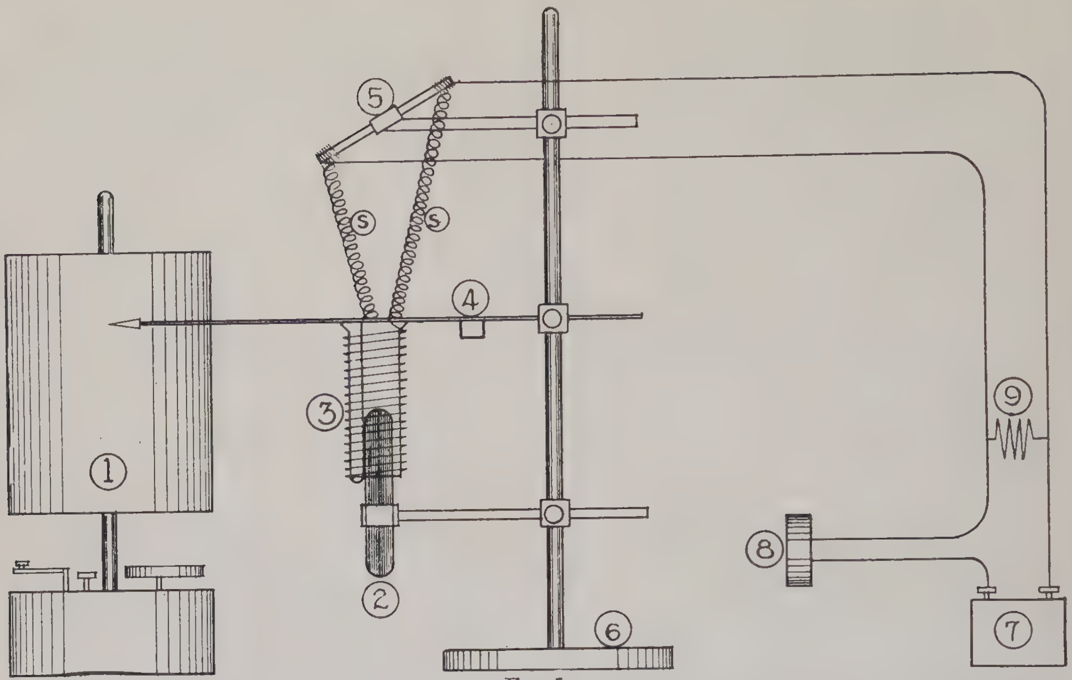


FIG. 1.

The Electro-Magnetic Sphygmograph. 1. Drum. 2. Permanent magnet. 3. Coil on bisected test tube. 4. Writing lever. 5. Horizontal bracket. 6. Stand. 7. D.C. battery. 8. Microphone. 9. Resistance. S. Suspension springs.

the wire being coiled somewhat tightly to serve as an elastic mounting of the tube and its coil. The ends of the wire constituting the coil then serve as supports as well as terminals through which the current passes. One pole of the D.C. battery is connected to one end of the wire mounting; the other battery pole is connected through the microphone to the other terminal. A 5 ohm resistance is placed in parallel to act as a damping device. A light heart lever is mounted upon the iron stand and its writing point is brought against the Kymograph beyond the coil upon which it rests. The actual transcription results from the following considerations:

With increasing pressure of the artery, the decreasing resistance of the microphone permits a proportionally greater current ($I = E/R$) to be delivered to the coil. This greater current causes a proportionally increased magnetic flux through the recording coil but in the opposite direction to the field of the permanent magnet. This opposition literally lifts the coil and gives an upswing to the writing point and produces on the actual sphygmogram and anacrotic limb. Since the drop off of the throb creates differential currents through the microphone, also the catacrotic limb with its most minute variable phases are faithfully transcribed. The essential details of the assemblage is shown here in Fig. 1.

15570

Reproductive Processes in Proteus Cultures.*

L. Dienes.

From the Department of Pathology and Bacteriology, Massachusetts General Hospital, and the Robert W. Lovett Memorial, Harvard Medical School, Boston, Mass.

A different and more complex form of reproduction than binary fission has been observed in Gram-negative bacteria.[1] In exceptional strains of several species most of the bacteria swell up into large round bodies which in turn develop into regular bacteria or a peculiar variant form. Sometimes bacteria form inside the large bodies; sometimes the latter disintegrate into irregular fragments which after a few divisions assume the usual bacterial form. The peculiar variant form consists of small soft granules which have a tendency to invade agar and to swell into large round bodies. This variant is similar in appearance and properties to the pleuropneumonia group of organisms. Following Klieneberger, it is designated with the letter L. Klieneberger[2] discovered this type of colony (L_1) in cultures of Streptobacillus moniliformis. The L_1 variant isolated from this species is easily reversed to its parent form during a varying length of time after isolation. It is serologically similar to the parent form. The L_1 appears to be an intermediary link in the development of bacteria from the large bodies.[3] The reversion of L forms into the parent bacterial forms has not been observed in other species. The complexity of these reproductive processes and their similarity in different species makes it unlikely that they are degenerative. It has been pointed out elsewhere[3] that the L forms present similarities to certain yeast forms which Winge[4] obtained by cultivation of single spores of an *ascus* and which he designated "haploform."

All bacteria in which these processes have been observed were isolated from pathological processes or from mucous membranes. On artificial media the bacteria were not in their natural environment. For this reason it was thought that a study of saprophytic organisms, to which artificial media are not so foreign, might afford some information as to the significance of these reproductive processes. The study of Proteus was undertaken because large bodies are present in small numbers in the spreading halos of most strains. There is some uncertainty as to whether the Proteus strains used in the present study are truly saprophytic because they were isolated from urine and stool specimens. However, Proteus does not seem to be adapted to a true parasitic life, and its natural habitat is outside the living organism.

The Proteus cultures develop in consecutive phases of growth *in situ,* and of spreading. The bacteria are markedly different during these phases. The growth starts in a lightly inoculated plate as small bacilli with no tendency to spread. After the colonies reach a certain stage of development the bacilli at the edge of the colonies grow into filaments abundantly provided with flagellae and start to move actively on the surface of the medium. These actively moving filaments produce the spreading halo. When the halo reaches a certain size the filaments break into short rods and multiply as such without further spreading. The filaments appear again after a certain interval, and the spreading is resumed. Filaments usually are not produced in liquid media. Of 30 strains studied, 25 grew in spreading cultures; 5 strains lost this characteristic soon after isolation.

Accidental observations suggested that large bodies are more numerous in mixed cultures of different strains. Experiments made to study this point brought unexpected results.

When broth cultures of certain strains are

---

* The expenses of this investigation have been defrayed in part by a grant from the Commonwealth Fund. Publication 95 of the Robert W. Lovett Memorial.

[1] Dienes, M., and Smith, W. E., *J. Bacteriology,* 1944, **48**, 125.

[2] Klieneberger, E., *J. Path. Bact.,* 1935, **40**, 93.

[3] Dienes, L., to be published.

[4] Winge, O., *Compte. r. Trav. Lab., Carlsberg,* 1935, **25**, 72.

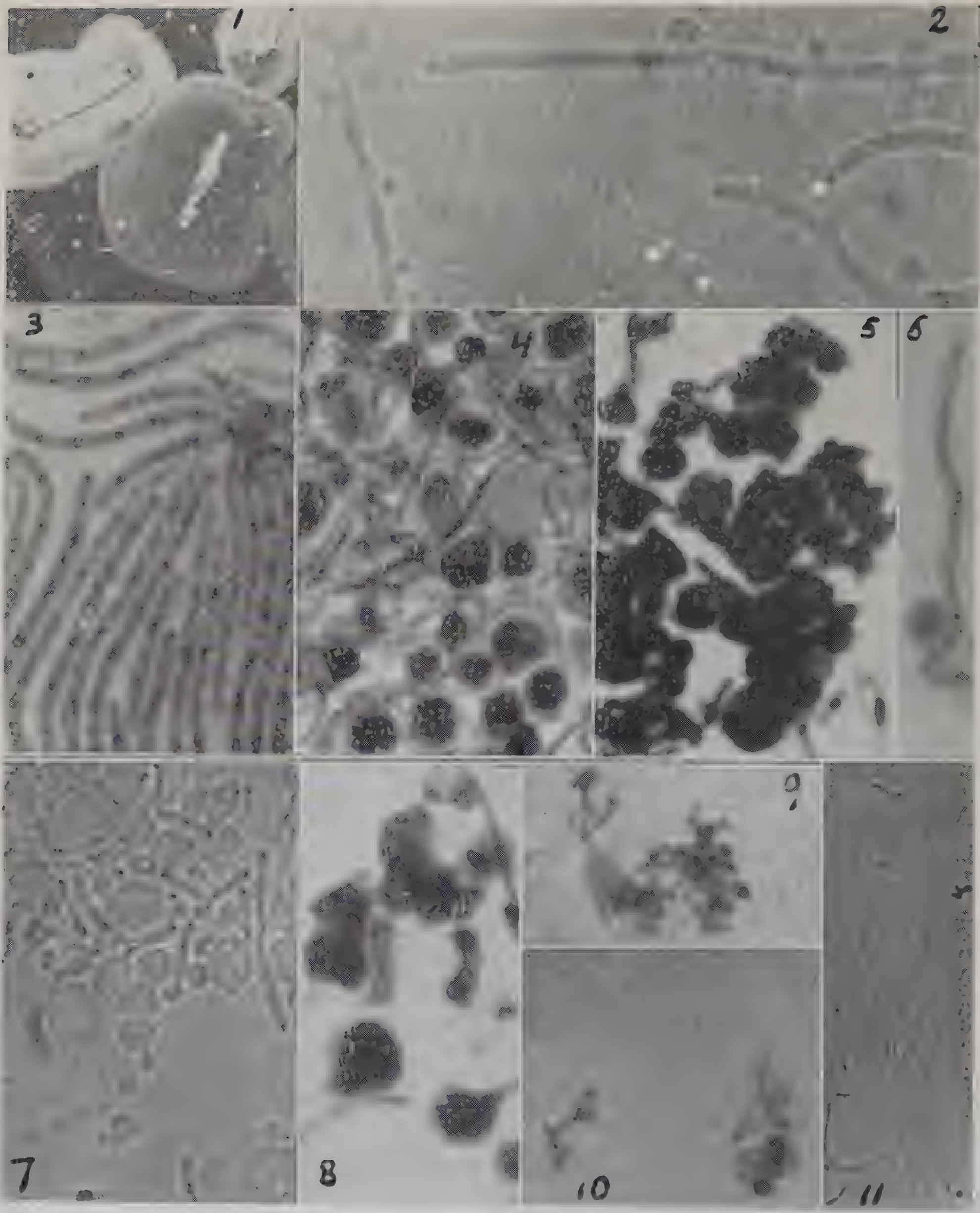


Fig. 1

Photograph 1. The meeting of spreading halos of strains 3 and 14 on a blood agar plate. Note the line of demarcation between the halos.

2. Filaments of strain 3 transferred to distilled water. Their content oozes out as droplets attached to the filaments. Unstained, × 3000.

3. Spreading filaments of a halo. Wet agar preparation stained with methylene blue. × 3000.

4. Large bodies at the meeting line of 2 halos. Most of the filaments are attached to the large bodies and are shrunken. Dry impression preparation after agar fixation stained with methylene blue. × 3000.

5. Large bodies on the surface of agar inoculated with the spreading filaments of 2 halos. Incubation, about one hour. The deformation of large bodies is the first sign of their development into usual bacterial forms. Only few bacterial filaments remained visible between the large bodies. Giemsa staining after agar fixation. × 3000.

6. A bacterial filament at the meeting site of 2 halos with a large and a tiny round body attached to it. Wet agar preparation stained with methylene blue. × 3000.

7. Large bodies developing on agar inoculated with filaments from 2 halos. Only few bacteria retained their usual shape. Unstained, × 2000.

8. Large bodies enlarged and deformed from a culture similar to that represented in Photograph 5. Lighter staining with methylene blue allows better recognition of the shape of the large bodies. × 3000.

9 and 10. L type of growth originating from large bodies produced in tapwater and transferred to agar. The large bodies are deformed and stain unevenly. In the left lower corner of Photograph 10, the small bacillary and round forms of the L type of growth are fairly well visible. Dry agar preparation stained with methylene blue and azur. × 3000.

11. Spreading filaments transferred to distilled water. Nearly all filaments produced large bodies. Unstained. Moderate magnification, × 1000.

mixed and planted on agar, large bodies appear in clusters situated at the edge of and between the originally developed colonies when the spreading starts. The large bodies are attached to the bacterial filaments (Photographs 2 and 6, Fig. 1). The interaction between strains can be observed most clearly when strains are planted separately on agar and the zone where the spreading halos meet is examined. It is usually necessary to examine several oil immersion fields in a spreading halo to find one large body. On the other hand when 2 halos meet, most of the filaments are transformed into large bodies, and are thus immobilized within a short time (10 to 30 minutes). Photographs 3 and 4 illustrate the organisms in a halo and in the meeting zone of 2 halos, respectively. In Photograph 3 there are long, winding filaments which appear segmented when stained with methylene blue. In Photograph 4 most of the organisms are in the form of large bodies. The visible filaments are shrunken and in most cases are attached to large bodies. Large bodies are also abundantly produced when agar blocks containing the halo of one strain are cut out and superimposed on the halos of another strain.

The production of large bodies in mixed cultures depends on 2 fundamental conditions: the strains must be appropriate, and spreading halo must meet spreading halo.

Filaments spreading from 2 colonies of the same strain exert no influence on each other when the halos meet. Two of the 30 strains studied (3 and 7) produced large bodies with all the others. They did not influence each other. The next best strain was tested with 12 others and produced large bodies with 8, including strains 3 and 7. The number of strains examined is not sufficient to indicate whether there are groups of strains which react with other groups or whether some strains are more apt to produce large bodies than others.

The meeting of the cultures produces no effect if one of them is in the small bacillary form, the stage preceding spreading. Only motile filaments are transformed into large bodies and only when they meet motile filaments of another strain. Whether the filaments of both strains are involved cannot be determined with certainty. When the filaments of one strain are shorter than those of the other, large bodies are attached both to the longer and to the shorter filaments. This observation suggests the participation of both strains. The fact that at certain sites nearly all filaments have large bodies attached to them also suggests that both strains are involved.

The mixing of strains is not the only way to induce the transformation of spreading filaments into large bodies. Exposure of the culture to cold or transfer into tap or distilled water or into broth containing small amounts of $HgCl_2$ often induce this transformation. If the cultures containing fresh halos are kept overnight in the refrigerator, most filaments develop into large bodies within a short time after the cultures are warmed to room temperature. Large bodies appear also within a few minutes if the spreading filaments of some strains, especially those of strains 3 and 7, are transferred into tapwater. The mechanism by which the large bodies are produced can be most clearly observed in tapwater. They are produced by a process which has long been known as "plasmoptysis." The contents of the filaments ooze out and remain attached to the filaments as small droplets. A droplet may increase rapidly in size and may develop within 1 or 2 minutes into a full-sized round body. Meanwhile the filament shrivels. It is thus apparent that most of the content of the filaments flows out to produce a large body. Large bodies are produced in a similar way in mixed cultures or after refrigeration. During this process, although the filaments shrink visibly, they retain their motility both in tapwater and on the surface of agar and drag their attached large bodies rapidly about among other bacteria. The large bodies are bent and deformed by collisions; they may be drawn through narrow passages. It is therefore apparent that the large body is a resistant elastic structure firmly attached to the filament. It very rarely happens that a large body is destroyed by mechanical injury. In this case it disappears without leaving a trace.

In tapwater, filaments of different strains produce large bodies in varying proportions. In strains 3 and 7 many filaments are transformed into them. In most strains only a few large bodies are so produced. Transfer from agar into broth or ascitic fluid also induces the production of a few large bodies. The small bacilli of the first growth stage are not affected by exposure to cold or to tapwater.

Most filaments of strain 3, when transferred into broth containing 0.01% $HgCl_2$ were transformed into large bodies, but this concentration prevented further growth. When the concentration of $HgCl_2$ was 0.005 and 0.0025% the small bacilli remained motile, continued to multiply and many filaments produced large bodies. They were produced, as in tapwater, by "plasmoptysis" • $HgCl_2$, many other toxic salts and penicillin also induce the formation of large bodies in various other bacterial species. Phenol has no similar effect. Large bodies produced from Proteus or other bacteria under these conditions are not viable.

The viability of large bodies and the type of growth which they produce varies according to the circumstances under which they are produced. Most of the large bodies produced in mixed cultures and after refrigeration will grow and change back within a short time to the usual small bacteria. This process occurs in a manner similar to that observed in Bacteroides.[1] The large bodies increase in size and their contour becomes irregular. They fractionate and after further growth and division their parts regain normal bacterial form. Photographs 5 and 7 show the enlarged irregular bodies which represent the first step in the retransformation into bacteria. It is apparent in the picture that nearly all large bodies are developing. At certain sites hardly any filaments survive, and the new growth originates from large bodies. At first growth is retarded at the meeting of spreading halos. This retardation is temporary, and the large bodies soon resume multiplication. The development of tiny bacterial colonies from large bodies has been observed in slide cultures. It seems unnecessary to publish a photographic record of this process because this has already been done in the case of Bacteroides. Occasionally, as in other species, L type colonies develop from these large bodies.

The development of large bodies produced in tapwater is not uniform. Most of them do not develop when transferred to agar. A few may produce the L type growth. The morphology of the parent form is produced only on rare occasions. Tiny L type colonies growing from large bodies are illustrated in Photographs 9 and 10. They reach their highest development after 8-12 hours' incubation, and rarely grow to larger size than the colony shown in Photograph 8. They are covered by bacterial growth originating from filaments not transformed into large bodies and disappear. Their isolation in pure culture has not been successful. The L type growth in Proteus is very similar to the corresponding growth in *E. coli.*[5]

The differences in the development of the large bodies produced in mixed cultures and after exposure to refrigeration on one hand, and in tapwater and in broth containing $HgCl_2$ on the other hand are so marked that there must be important differences between the large bodies themselves. It has already been mentioned that large bodies produced by $HgCl_2$ did not develop when transplanted. The author previously has observed that the large bodies produced in cultures of various bacteria are viable only when they appear during the natural development of the cultures. When toxic influences induce the formation of large bodies, they probably awaken a latent tendency in the bacteria in an imperfect manner, but they do not produce viable large bodies and are not able to bring the process to its normal conclusion. The difference between the large bodies produced by different means in Proteus is the more remarkable because they seem to be produced in mixed cultures by dissolved antagonistic substances just as they are in a medium containing $HgCl_2$ or penicillin. It was not possible to substantiate the natural first impression that large bodies developing in mixed cultures are sexual forms. Conjugation is not apparent in mixed cultures either between filaments or between large bodies. No other formal elements which could indicate a sexual process were visible in preparations stained with Giemsa or with flagellar-staining methods. On the other hand, the antagonistic effect of the strains on each other is apparent. Halos starting from colonies of different Proteus strains do not penetrate into each other. Even after several days of incubation a sharp line of demarcation persists between the areas covered by different strains. The halos sometimes stop at a short distance from each other without coming into contact, indicating the diffusion of antagonistic substances into the medium. Meanwhile extension proceeds in other directions. Large bodies are not produced without contact between filaments, but this fact could be explained by differences in concentration of the antagonistic substances. The present observations suggest that large bodies are produced in mixed cultures by dissolved substances and that viability distinguishes such large bodies from those produced by toxic influences.

---

[5] Dienes, L., *J. Bacteriology*, 1942, **44**, **37**.

There are observations which suggest as a possibility that the influence of one strain on the other may be more than a simple antagonism. Avery's[6] observations on the interchange of capsules between pneumococcus strains suggest that hereditary characteristics in bacteria can be transmitted by dissolved nuclear substances without morphological organization. If this is true, processes corresponding to sexuality in higher organisms may be possible in bacteria without an exchange of organized nuclei. The fact that in Proteus 2 strains are involved in the production of large bodies presents an opportunity to study whether an exchange or redistribution of hereditary characters occurs during this process.

Summary. The complex reproductive processes formerly observed in certain Gram-negative bacteria have also been observed in Proteus cultures. In this species they presented several distinctive characteristics. The large bodies in every case previously studied were produced by gradual swelling of the bacteria. In Proteus they are produced by a process known as "plasmoptysis." The large bodies so produced possess similar physical properties and show reproductive processes similar to those formed in other species by swelling of the bacteria.

Another characteristic of Proteus is that the bacteria from which the large bodies develop differ considerably from the bacteria usually present in the culture. Large bodies develop only from spreading filaments actively motile on solid media. In almost every culture these filaments produce an occasional large body, and most of them are transformed into large bodies when the spreading halo of one strain meets the spreading halo of another appropriate strain. The large bodies so produced are viable and within a short time are transformed into regular bacteria. Exceptionally they produce the L type of growth. There is a great variation in the reaction of different strains to each other.

The spreading filaments show a tendency to "plasmoptysis" and production of large bodies under various adverse conditions, for example, when they are subjected to refrigeration or when they are transferred into tapwater or into broth containing $HgCl_2$. The large bodies produced by refrigeration are apparently fully viable. Those produced by tapwater are only occasionally viable, and when they germinate they usually produce L type colonies. Large bodies produced by

---

[6] Avery, O. T., MacLeod, C. M., and McCarthy, M., *J. Exp. Med.*, 1944, **79**, 137.

$HgCl_2$ are not viable. The difference in viability constitutes an important difference between the large bodies produced by the interaction of the 2 strains and by refrigeration on one hand, and by toxic influences on the other hand. The meeting of the 2 strains induces a complex reproductive process consisting of several consecutive steps. After a short delay vigorous multiplication is resumed. It is not likely that this process is simply degenerative.

No union was observed between cells at the meeting of 2 strains and the present observations do not support the supposition that the interaction between the strains is sexual.

15571

Differences in Culturability, Infectivity and Pathogenicity of Human Strains of *Endamoeba histolytica*.*

ERNEST CARROLL FAUST, ILSE HEILBRUNN, RUTH LEWIS, AND MARY LU MURRAY.
From the Department of Tropical Medicine, Tulane University of Louisiana, New Orleans, La.

Introduction. Clinical and experimental data have provided a wealth of divergent views concerning the ability of *Endamoeba histolytica* to produce disease. In man and experimental animals there is a wide range of clinical states in amebiasis, from a fulminating infection to an essentially symptomless condition. Both the intrinsic pathogenicity of the particular "strain" of the organism and the host's general threshold of resistance to infection contribute to infectability and the degree of pathology produced.

Since the accumulated knowledge fails to explain many theoretical and practical questions concerning *E. histolytica* and its host, a renewed attack has been made on the problem. The particular objective of the present study consists in an evaluation of culturability, infectivity and pathogenicity of different "strains" of the parasite.

Material and Technics. Eighteen persons have served as sources of the parasite. The youngest was 2 years and the oldest 59 years old. Twelve were males, 5 females and the record on one was not available. Six were white, 12 were Negroes and one was an Indian. All but one, a Honduran, were American-born and the majority were natives of Louisiana who had never left the state. Twelve persons sought medical help because of colitis, including diarrhea and chronic dysentery. Two individuals in the series were suffering from bronchopneumonia. One patient complained of rheumatism and one was suffering from oxyuriasis. A food handler undergoing routine coprological diagnosis had no complaint. The clinical diagnosis of one was not obtained. Of the entire group 7 passed formed stools from which cysts of *E. histolytica* were obtained and 4 submitted unformed stools containing motile trophozoites of the organism. In 5 instances proctoscopic material was obtained for inoculation. In one case a purgative specimen was used. In another, a dysenteric stool was cultured in the hospital laboratory. In 11 instances cysts of trophozoites in freshly passed stools were utilized.

The basic culture medium consisted of an egg-slant base, made of a coagulated emulsion of whole egg and Ringer's solution, with liquid overlay of physiological salt solution containing 0.5% crude liver extract (Lilly). After tubing, a small amount of specially milled rice starch, furnished by Doctor Charles W. Rees of the National Institute of Health, Bethesda, Md., was sterilized and planted in the overlay. Formed stool specimens provided cysts, which were concentrated by the zinc sulfate centrifugal floatation technic, thoroughly washed in distilled water and then seeded in the tubes. Small amounts of mucus

---

* Assisted by a research grant-in-aid, National Institute of Health, Bethesda, Md.

or feces and mucus from the unformed or liquid stools, proctoscopic material and the purged sample were planted directly in the medium. Culture tubes were incubated at 37°C and transfers were usually made every 48 hours, although some "strains" required a different interval for subculturing and others varied considerably in their growth periods from week to week. The optimum transfer period was determined only after prolonged observation with each "strain." Routinely at the appropriate time for transfer the tube was opened and under aseptic conditions a small measured amount of the liquid at the base of the overlay was drawn up into a sterile pipette and transferred to a fresh culture tube. A second measured amount was then removed from the first tube and placed on a microscopic slide provided with a 22 mm square coverglass, for observation and for count of the amebae. When fewer than 10 amebae were found in the entire preparation, growth was designated as very poor; when an average of one to 5 appeared in each low-power field, it was designated as poor, 5 to 20 as fair, 20 to 50 as good, and more than 50 per low-power field, as excellent. At times no amebae were seen in the cover-glass preparation but subcultures proved positive. Moreover, under conditions which did not vary, all cultures sometimes developed better than average, while at other times great difficulty was experienced in maintaining even those which usually exhibited luxuriant growth. During the period of this study encystation occurred only sporadically and in scant amount.

The experimental animals consisted of young kittens, mature cats, puppies from one to 5 months old and dogs about 6 to 9 months of age or older. Although puppies proved to be as infectable as kittens, they were not readily obtained. Cats were tested only incidentally. Older dogs were used to maintain "strains" over a period of several months. Confirming the experience of previous investigators, the present workers found kittens to be relatively satisfactory for acute experiments, both because of the ease of infectability and because of the rapid development of lesions. Early in the study it was decided to employ a minimum of 5 kittens to test the infectivity and pathogenicity of each "strain." Except for 3 "strains" (Nos. 2, 9 and 12), the minimum goal was reached and in several instances considerably exceeded.

The senior author[1] has described a practical technic for inoculating dogs *per anum* by means of a long glass tube to which a rubber bulb is attached, so that an inoculum with amebic trophozoites can be deposited in the cecum and usually into the posterior segment of the ileum, the anteriormost level of the bowel where amebic lesions develop. The technic referred to provides an opportunity for the amebae to make contact with the intestinal mucosa and to penetrate the tissues without trauma to the bowel and without the hazard of shock and lowered resistance attendant on the implantation of the inoculum by needle through the wall of the posterior segment of the ileum following laparotomy.[2] Although the nonflexible glass inoculation tube can be used in the kitten without danger of injury to the delicate bowel wall, a very satisfactory substitute consists in a No. 10 or 12 flexible rubber catheter, to which a hypodermic syringe is attached.

In 5 instances ("strains" No. 6, 7, 8, 10, 15) cysts were concentrated, washed, and were fed to kittens, cats and puppies to initiate infection. In 4 instances ("strains" No. 11, 13, 17 and 18) trophozoites obtained directly from human sources were inoculated intracecally. In these 9 "strains," as well as in all others except No. 9 and 12 where material was too scant, coincident or subsequent inoculations were made intracecally in other hosts with cultured trophozoites.

Pathogenicity has been evaluated by 2 types of data, clinical and postmortem. An estimate of the effect of the amebae on their experimental hosts during life was based on the behavior of the animal compared with the preinoculated condition, loss of appetite, loss of weight, condition of the fur, number and type of bowel evacuations and the relative prevalence of amebae in the stools. On this basis an attempt was made to estimate the damage to the host in categories of +, ++, +++ and ++++.

---

[1] Faust, E. C., *Porto Rico J. Pub. Health and Trop. Med.*, 1931, **6**, 391.

[2] Meleney, H. E., and Frye, W. W., *Am. J. Hyg.*, 1933, **17**, 637.

Only kittens will be considered in the postmortem study, since they alone were employed in numbers sufficient for statistical use. A total of 93 kittens came to necropsy or were sacrificed. Two of these confirmed clinical and antemortem laboratory evidence that the particular animals were refractory to infection. In one which had been positive some weeks earlier there was no indication of infection, suggesting that spontaneous recovery had occurred. No autopsy was performed in 11 instances and in 3 kittens advanced postmortem changes made careful pathological examination useless. The 76 remaining in the series were available for full study. The workers were from time to time assisted by Doctor Guillermo M. Carrera, of the Department of Pathology and Bacteriology, Tulane University.

Eight levels of the bowel potentially involved in amebic ulceration were inspected separately in each kitten, *e.g.*, posterior segment of the ileum, cecum, appendix, ascending colon, transverse colon, descending colon, sigmoid colon and rectum. The degree of involvement of each segment was then recorded on the basis of naked eye and dissecting-microscope inspection. In each autopsy the presence of amebae recovered from the lesion or free in the bowel was also recorded. The following symbols were employed to designate the degrees of pathogenicity: one to 10 small shallow amebic ulcers per segment, +; 10 to 25 distinct shallow ulcers or a smaller number of deeper ones, ++; deep ulceration with some evidence of confluence, +++; extensive confluent lesions over essentially the entire segment of the bowel, ++++. These estimates were made for each of the 8 segments of the 76 autopsied animals. "Strain" No. 5, with 10 kittens in the series, will illustrate how the amount of tissue damage was recorded and evaluated (Table I). Each + was assigned a numerical value, with + as one, ++ as 2, +++ as 3 and ++++ as 4. Thus the maximum total units for an entire length of intestine with severest involvement would be 32. Then, the

TABLE I.

Evaluation of Tissue Damage (with Percentage Involvement) in 10 Kittens Experimentally Inoculated with "Strain" No. 5, *Endamoeba histolytica*, as Studied at Autopsy.

	Ileum	Cecum	App.	Asc. colon	Trans. colon	Desc. colon	Sigmoid colon	Rectum	Total unit value	Pathogenic index
	—	+(20) ++(30) +++(20)	+(20)	+(20) +++(10)	+(30) ++(20)	+(20) ++(10) +++(10)	+(30) ++(20) +++(10)	+(40) ++(30) +++(10)		
Total % involvement		Σ = 70	Σ = 20	Σ = 30	Σ = 50	Σ = 40	Σ = 60	Σ = 60		
Unit value		1.4	0.2	0.5	0.7	0.7	1.0	1.3	5.8	5.8 ÷ 32 = 0.18

TABLE II.
Summary of Data on Culturability, Infectivity and Pathogenicity of 18 "Strains" of *E. histolytica* From Human Sources.

Source and type of inoculum	Culturability	Infectivity	Pathogenicity: Clinical estimate	Pathogenicity: Pathogenic index
Proctoscopic;	Very poor			
trophozoites	No. 2	Low ?	+ ?	
Idem.	" 9	" ?	?	
"	" 12	" ?	?	
	Poor			
Stool; cysts	No. 7	"	?	
Purge; trophozoites	" 11	High	++++	0.08
Proctoscopic; "	" 14	"	+++	0.23
	Fair			
Stool; "	No. 3	"	++	0.17
Proctoscopic; "	" 13	"	++++	0.11
Stool → culture; trophozoites	" 16	Moderate	+ to ++++	0.15
	Good			
Stool; cysts	No. 1	"	+	0.11
" ; trophozoites	" 4	"	+	0.02
" ; cysts	" 6	Low	+++	0.13
" ; "	" 8	"	+	0.08
" : "	" 15	Moderate	++	0.11
" ; trophozoites	" 17	"	++++	0.12
" ; "	" 18	"	++++	0.28
	Excellent			
Stool; cysts	No. 5	High	++	0.18
" ; "	" 10	Moderate	++	0.12

total number of units for all members of the series divided by the number of animals in the series equals the average total unit value per animal (*i.e.*, for "strain" No. 5 = 5.8). However, since it is more convenient to evaluate and compare pathogenic indexes as percentages of 1.0, the average total unit values per animal have been divided by 32. Thus, for "strain" No. 5 the pathogenic index was found to be 0.18.

Presentation of Results. The most important data on culturability, infectivity and pathogenicity of the 18 "strains" studied have been assembled in Table II.

There is no evidence of a consistent correlation between the *in vitro* and *in vivo* experiments, yet the following relations have been found to exist. (1) On the whole, cysts and trophozoites of *E. histolytica* obtained from stools provided better material for culture than did trophozoites from proctoscopic material. (2) The majority of "strains" which developed poorly in cultures were not satisfactory for infecting kittens and with one notable exception (No. 14) failed to produce significant lesions in these animals; conversely, the majority of "strains" which developed fairly well to abundantly in culture proved successful for infecting kittens, although these strains did not necessarily produce extensive tissue damage in the test animals. (3) Clinical evidence is at times misleading for evaluating the extent and degree of amebic lesions produced in the kitten.

In "strains" No. 6, 7, 8, 10, 11, 13, 15, 17 and 18, in which the ameba was inoculated both from original source material (cysts by mouth and trophozoites intracecally) and following growth in the test tube, there was no detectable difference in the rate of infection or the lesions produced from the 2 sources of inocula. In some tests, months after the original material ceased to be available, the cultured organisms proved as satisfactory for inoculation as earlier in the study. In other words, growth in the test tube did not reduce or enhance infectivity and pathogenicity. "Strain" No. 13, which from the start was highly infective, failed to grow in culture from human source material. Later it became established from amebae obtained postmortem from kittens, although it has never

TABLE III.
Summary of Consolidated Postmortem Data on Amebic Involvement of Intestine in 76 Experimental Kittens.

Degree of involvement	Number and % of lesions at different levels							
	Ileum	Cecum	App.	Asc. colon	Transv. colon	Desc. colon	Sigmoid colon	Rectum
Negative	76 (100.0)	33 (43.4)	62 (81.6)	56 (73.7)	57 (75.0)	54 (71.0)	39 (51.3)	41 (53.9)
+		19 (25.0)	11 (14.5)	9 (11.7)	12 (15.8)	8 (10.6)	16 (21.2)	12 (15.8)
++		14 (18.4)	3 (3.9)	4 (5.3)	5 (6.6)	10 (13.2)	14 (18.4)	15 (19.8)
+++		6 (7.9)		6 (7.9)	1 (1.3)	3 (3.9)	5 (6.6)	5 (6.6)
++++		4 (5.3)		1 (1.3)	1 (1.3)	1 (1.3)	2 (2.6)	3 (3.9)
Total	0	43 (56.6)	14 (18.4)	20 (26.3)	19 (25.0)	22 (29.0)	37 (48.7)	35 (46.1)

until recently exhibited vigorous culture growth. "Strains" No. 17 and 18 were obtained from a sister and brother and quite possibly may be identical. Experience with them has been parallel except that "strain" No. 18 provided a much higher average pathogenic index in the experimental animals.

In the series of kittens for which complete autopsies were performed 54 (71.1%) showed typical amebic lesions, which varied in degree from a solitary pinpoint ulcer to extensive involvement of the large bowel. The regional distribution of the lesions, together with the number and the estimated degrees of pathogenicity for each of the 8 segments examined, are summarized in Table III.

Except for the absence of lesions in the ileum and on the ileocecal valve, the levels of greatest frequency and intensity of amebic ulceration are those which previous investigators have discovered at autopsy of human beings and experimental animals, namely that the cecum is the region of greatest involvement, followed by the rectum. This is particularly true of the early acute infection, whereas in prolonged chronic amebic colitis there is a tendency for secondary amebic lesions to develop in the rectal area following invasion of amebae which have previously propagated at the cecal level.

In the 2 "strains" of this series with the lowest pathogenic indexes (No. 4 and 8) there were no lesions discovered in the ileum, ascending or transverse colon. With "strain" No. 4 patent lesions were developed in only one of 6 animals. These consisted of first degree damage in the appendix and second degree in the rectum, while no other levels were invaded. In the 7 kittens employed in a study of "strain" No. 8, 2 had first degree lesions in the cecum, one had first degree lesions in the ascending colon and descending colon, 3 had first degree and one had second degree lesions in the sigmoid colon, and 2 each had first and second degree lesions in the rectum. The observations for "strains" No. 14 and 18, both with high pathogenic indexes, indicate much more extensive involvement. In "strain" No. 14, with 7 animals in the series, every level of the bowel from cecum to rectum was invaded; except for the appendix there were lesions of third or fourth degree in one or more kittens. This "strain" was also highly pathogenic for the cat, which is relatively refractive to amebiasis. In "strain" No. 18, with 5 kittens in the series, there was no evident pathology in the appendix but third or fourth degree lesions were demonstrated at the other levels in 2 members of the series.

In all kittens of the autopsy series antemortem-aspirated material had been obtained *per anum* from the intestine at most from one to 2 days before death or sacrifice of the

TABLE IV.
Summary of Records on *E. histolytica* and on Typical Amebic Lesions in the Intestine of Experimental Kittens.

E. histolytica			Coincidence of amebic lesions		
	No.	%		No.	%
Antemortem +, postmortem +	27	35.5	Present	26	34.2
			Not found	1	1.3
” +, ” —	15	19.7	Present	13	17.1
			Not found	2	2.6
” —, ” +	4	5.3	Present	4	5.2
			Not found	0	0
” —, ” —	30	39.5	Present	11	14.5
			Not found	19	25.0
Total antemortem +	42	55.3	Total present	54	
” ” —	34	44.7	” not found	22	
” postmortem +	31	40.8			
” ” —	45	59.2			
Grand total	76	100.0	Grand total	76	100.0

animal, to provide microscopic evidence of infection. In 47 cases (61.8%) trophozoites were demonstrated. Postmortem search for the organism was successful in 31 cases (40.8%). In 4 of this latter group amebae had not been demonstrated from antemortem stools or aspirates. Moreover, no lesions were found in one puppy and one adult dog inoculated with "strain" No. 18, but motile amebae were demonstrated both antemortem and postmortem. The relationship of amebae recovered antemortem and postmortem and of amebic lesions is summarized in Table IV.

An examination of Table IV indicates that in 27 kittens (35.5%) there was consistent microscopic demonstration of the amebae and of amebic lesions. In an additional 15 animals (19.7%) the organisms were found shortly before death; typical lesions were found in 13 of these but amebae were not demonstrated postmortem from bowel contents or superficial scrapings. On the other hand, in 4 cases no amebae were recovered antemortem, although they were demonstrated postmortem in association with typical lesions. A fourth category consisted of 30 kittens (39.5%) in which amebae were not demonstrated immediately before death or at autopsy; there were typical lesions in 11 of these but there was no gross evidence of damage in 19 animals. Among these 30 animals some had earlier provided specific microscopic evidence of infection. The data favor the view that in 25.0% of the 76 kittens studied postmortem an infection (*i.e.*, tissue invasion) had never developed, or, if it had existed earlier, the amebae had been expelled with spontaneous recovery and tissue repair. In the single instance in which amebae were demonstrated from the bowel contents both antemortem and postmortem but in which lesions were not found at autopsy there is the possibility that one or more minute inapparent cryptic ulcers were present. In the 2 animals in which there was antemortem but not postmortem demonstration of the amebae and no lesions were discovered at autopsy the infection had probably been spontaneously cleared.

Discussion. This study has provided considerable evidence that culturability, infectivity, and pathogenicity constitute 3 separate attributes of *Endamoeba histolytica*, and that they are not necessarily correlated with one another. A "strain" which grows well in culture (Table II) may be established in the intestine of a highly susceptible animal only with difficulty and following persistent trial. Conversely, a highly pathogenic "strain" which produces infection readily in the test animal, may be established and maintained in culture only after considerable effort.

A factor which will require careful experimental inquiry is the presence or absence of certain species of bacteria associated with the amebae in the inoculum utilized to initiate cultures. Until such information is available in rather complete form it will not be possible to rule this out as an extrinsic influence on initial and subsequent growth.

The present study has provided data indicating that the infectivity of a particular "strain" of *E. histolytica* and the associated infectability of a susceptible host are not always correlated with symptoms and lesions. In 2 "strains" which had a low rating for infectivity the degree of pathogenicity was high. Conversely, in certain "strains" with a high estimate of infection only incidental lesions developed in the series. Nevertheless, on the whole, "strains" which were established with difficulty in the experimental host produced only a few low-grade lesions, while those with medium to high infectivity caused moderate to severe pathology.

One of the most interesting developments in the present investigation has been the rather conclusive evidence (Table III) that the clinical estimate of the extent of the disease in the experimental host frequently fails to coincide with the data obtained at autopsy. At times the antemortem estimate is in excess of the tissue damage, while at times it is too conservative. This latter type of prognostication corresponds frequently with that of human cases of amebiasis, in which the pathologist at necropsy finds extensive involvement of the large bowel not indicated by antemortem records.[3]

Summary. Investigations have been undertaken in an attempt to reconcile some of the many conflicting opinions which have developed regarding the pathogenicity of *E. histolytica*. The problem undertaken and presented in this communication concerns the culturability, infectivity and pathogenicity of 18 different "strains" of the organism, obtained from persons in New Orleans. The hosts varied in age from 2 to 59 years. All but one were hospital or clinic patients, the majority of whom complained of some type of colitis. In 11 cases stools containing cysts or trophozoites were obtained; in 5 cases proctoscopic material was utilized, in one case a purgative specimen was secured and in one instance the inoculum consisted of material previously cultured in a hospital laboratory.

For culture work a solid egg-slant base was employed, with a liquid overlay of physiological salt solution containing 0.5% crude liver extract and special rice starch. Cysts were concentrated and washed before seeding the cultures; trophozoites in fecal or mucous samples were placed directly in the culture tubes. Kittens served as the principal animals to test infectivity and pathogenicity of each strain. A minimum of 5 kittens was employed. Pathogenicity was first estimated on the basis of clinical evidence, then carefully studied at autopsy. The length of intestine from the posterior level of the ileum through the rectum was considered as 8 separate segments and amebic invasion of the bowel separately assessed for each segment. Four degrees of pathogenicity were recognized and were designated as +, ++, +++ and ++++. Based on these symbols a pathogenic index was developed to afford comparisons between different series.

Of the 18 "strains" 6 proved difficult to grow in culture; 4 of these were unsatisfactory for animal work and were discarded. All other "strains" immediately or eventually provided fair to luxuriant cultures and with 2 exceptions were very satisfactory for infection experiments. Seventy-six kittens infected with 14 "strains" of *E. histolytica* provided autopsy material for evaluation of tissue damage produced by the amebae. The clinical estimates of pathogenicity at times failed to correspond with the postmortem findings.

It was not possible to predict the culturability of *E. histolytica* on the history of the patient from whom the inoculum was obtained, on the type of inoculum utilized or the stage of the parasite. Nor was culturability a necessary index of infectivity or pathogenicity. It is suggested that culturability, infectivity and pathogenicity are 3 separate characteristics of *E. histolytica*. A considerable amount of investigation is required to determine what extrinsic factors may influence the development of the parasite *in vitro* and in the susceptible host.

---

[3] Clark, H. C., *Am. J. Trop. Med.*, 1925, **5**, 157.

15572

A Method for Routine Determination of Streptomycin Levels in Body Fluids.*

ERNA ALTURE-WERBER AND LEO LOEWE. (Introduced by Albert E. Sobel.)

From the Department of Laboratories, Jewish Hospital of Brooklyn, Brooklyn, N.Y.

The rational employment of antibiotics for the successful treatment of infectious diseases involves the establishment of curative dosage schedules. Optimum dosage schedules are usually predicated on a correlation between the *in vitro* sensitivity of the infecting organism and maintenance of requisite blood levels as estimated by assays of blood and urine.

Exploratory clinical study of streptomycin thus far has indicated its effectiveness in the treatment of some diseases due to organisms not inhibited by penicillin. There is therefore need for a simple, reliable quantitative method of assaying streptomycin in body fluids for purposes of dosage standardization.

Most streptomycin assay methods hitherto described[1,2] employ as test organisms *Escherichia coli, Bacillus subtilis* or *Staphylococcus aureus* which are too insensitive for microdeterminations.

Donovick *et al.*[3] employing *Klebsiella pneumoniae No. 41* as test organism in a screened 1% tryptone broth have demonstrated inhibition by as little as 0.05 unit of streptomycin per ml of test broth. The technic described by these authors, however, seems to be too laborious to be applicable as a routine clinical procedure.

Our method for determining penicillin levels in body fluids[4] has proved consistent and reliable in more than 4000 assays. The technic was modified and adapted for similar estimations of streptomycin, utilizing as standard a streptomycin sensitive organism grown in appropriate media. *Klebsiella pneumoniae No. 41,*[†] which grows luxuriantly in simple media and is readily inhibited by streptomycin, seemed ideally suited as a test organism.

Klebsiella pneumoniae No. 41 was inoculated in various media having a wide range of growth-promoting properties in order to discover suitable conditions for culture. The basic concept of the method calls for the most simple, least-enriched medium which will insure adequate growth. Media for levels determination although relatively deficient, must compare favorably with routine nutrient media in its growth-promoting properties. Conditioned in this manner the test organisms are made more susceptible to the action of streptomycin thereby rendering the assay more sensitive. Furthermore a sensitive level test makes possible higher dilutions of body fluids thus eliminating or reducing (1) the growth stimulus of such fluids and (2) any serological interference due to contained native antibodies.

Growth of Klebsiella pneumoniae No. 41 in Different Media at Various Concentrations. All media investigated were first prepared in distilled water in 1% concentration and adjusted to pH 7.2, filtered hot and autoclaved at 15 pounds for 20 minutes.

Serial, 2-fold dilutions of the 1% media were prepared with sterile water (2 cc in each of 12 test tubes representing a concentration range of 1% to 0.0005%). The 12 tubes were each seeded with 0.1 cc of a 4-hour culture of *Klebsiella pneumoniae No. 41* grown in brain heart infusion broth and diluted to 10^{-4} with the medium employed in the test. The stock culture of *Klebsiella pneumoniae No. 41* was maintained

---

* Aided by a grant from the John L. Smith Fund for Medical Research of the Jewish Hospital of Brooklyn.

[1] Schatz, A., Bugie, E., and Waksman, S. A., PROC. SOC. EXP. BIOL. AND MED., 1944, **55**, 66.

[2] Waksman, S. A., and Reilly, H. J., *J. Infect. Dis.*, 1944, **75**, 150.

[3] Donovick, R., Hamre, D., Kavanagh, F., and Rake, G., *J. Bact.*, 1945, **50**, 623.

[4] Rosenblatt, P., Alture-Werber, E., Kashdan, F., and Loewe, L., *J. Bact.*, 1944, **48**, 599.

[†] We are indebted to Dr. G. Rake of the Squibb Institute for Medical Research for a culture of this organism.

TABLE I.
Growth of *Klebsiella pneumoniae No. 41* in Different Media at Various Concentrations.

	Media						
	Amigen	Yeast	Neopeptone	Bactotryptone	Savita	Bactopeptone	Beef extract
Growth	%	%	%	%	%	%	%
Absent (neg.)	.004	.004	.008	.008	.004	.016	.063
Retarded (1 +)		.008	.016	.016	.008, .016	.031	
Dense (4 +)	.008	.016	.031	.031	.031	.063	.125

on blood agar plates which were stored at 5°C. All tubes were incubated 18 hours and then tabulated turbidometrically for presence and density of growth. The gradations were designated as negative, 1+ or 4+, indicating respectively absent, retarded or dense growth.

It may be noted (Table I) that *Klebsiella pneumoniae No. 41* can be grown luxuriantly in any of the following relatively deficient media, namely, 0.008% amigen, 0.01% yeast extract, 0.031% neopeptone, 0.031% bactotryptone, 0.031% savita, 0.063% bactopeptone or 0.125% beef extract.

Streptomycin Sensitivity of Klebsiella pneumoniae No. 41 in Different Media at Various Concentrations. Serial 2-fold dilutions of streptomycin‡ were set up in 1%, 0.5%, 0.25% and 0.125% concentrations of savita, bactotryptone and neopeptone, each concentration in 12 tubes covering a streptomycin range of one unit to 0.0005 unit per ml with a 13th tube as control. Each tube was seeded with 0.1 cc of a 10^{-4} dilution of a 4-hour culture of *Klebsiella pneumoniae No. 41* grown in brain heart infusion broth. All tubes were incubated for 18 hours and then read for inhibition or growth.

The results (Table II) clearly demonstrate that media of deficient nutritional value render *Klebsiella pneumoniae No. 41* more sensitive to the *in vitro* action of streptomycin. The metabolic changes so induced conceivably make the organisms more susceptible to streptomycin. Studies of the extent of metabolic interference with the bacterial cell caused by continuous propagation in deficient media are under investigation.

TABLE II.
Streptomycin Sensitivity of *Klebsiella pneumoniae No. 41* in Different Media at Various Concentrations.

		Reaction to streptomycin—units per ml	
Media	%	Inhibition	Growth
Neopeptone	1.	.25	.125
	.5	.125	.063
	.25	.0625	.032
	.125	.016	.008
Bactotryptone	1.	.25	.125
	.5	.0625	.032
	.25	.016	.008
	.125	.004	.002
Savita	.5	1.0	.5
	.25	.5	.25
	.125	.125	.063

Method of Determining Streptomycin in Body Fluids. The media used in our current standard levels determination are 0.5% and 0.25% neopeptone which meet the requirement of being simple and relatively deficient though still capable of sustaining adequate growth of the test organism.

Sufficient clear serum for the assay can be obtained from 10 ml of blood. Sera under investigation are stored at 5°C until used at which time they are diluted 1:10, 1:20, 1:40 or 1:100 in the test medium. If levels between 0.16 unit per ml and 1.6 units per ml or higher are expected 0.5% neopeptone is employed. If a more sensitive endpoint is desirable the assays are performed with 0.25% neopeptone.

Aliquots of urine specimens are Seitz filtered as soon after voiding as possible and

---

‡ We wish to thank Mr. John L. Smith of the Charles Pfizer Company, who supplied the streptomycin used in this study.

TABLE III.
Diagram of Test.

	Undiluted or diluted serum									
Amt of serum	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1.0
” ” broth	0.9	0.8	0.7	0.6	0.5	0.4	0.3	0.2	0.1	0

To each tube add 1 ml neopeptone broth seeded with *Klebsiella pneumoniae No. 41* (4-hour culture diluted 10^{-4}).

	Standard: 0.8 units/ml									
Amt of standard	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1.0
” ” broth	0.9	0.8	0.7	0.6	0.5	0.4	0.3	0.2	0.1	0

To each tube add 1 ml neopeptone broth seeded with *Klebsiella pneumoniae No. 41* (4-hour culture diluted 10^{-4}).

stored at 5°C until processed. For assay they are diluted 1:10, 1:100, 1:1000 or, occasionally, 1:10,000 in test broth. The dilution factor for urine is predicated on the anticipated concentration in the urine, the urinary excretory curve being directly related to the time of administration and dose of streptomycin.

Sera are pipetted into 100 mm × 13.5 mm tubes in 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1 cc amounts.

A parallel series of tubes containing a standard solution of streptomycin is also prepared. From a stock standard solution stored at 5°C secondary standards containing 0.8 unit per ml are set up in test media, serum and urine. These standards are dispersed into test tubes as follows:

0.1 ml to 1 ml as in the diagram of the test (Table III). To all tubes sufficient neopeptone broth is added to total 1 ml. Two control tubes each containing 1 ml of broth are added to the test. To each of the tubes 1 ml of neopeptone broth seeded with *Klebsiella pneumoniae No. 41* is added, making a total of 2 ml in each tube. The seeded broth is prepared as usual by diluting a 4-hour culture 10^{-4}.

All the tubes are incubated at 37°C overnight (18 hours). Slight growth is disregarded. The readings are very clear and definite as between significant growth and inhibition.

Under the conditions of the test it requires 0.16 unit of streptomycin in 0.5% neopeptone and 0.032 units in 0.25% neopeptone to inhibit *Klebsiella pneumoniae No. 41* per 2 ml. This means that the test organism has a streptomycin sensitivity of 0.08 unit per ml in 0.5% neopeptone and 0.016 unit in 0.25% neopeptone.

In the calculation streptomycin levels are converted into units per ml of body fluid. For example, if 0.5 ml serum is required to inhibit the growth of the test strain and if 0.4 ml of serum permits growth and the test was performed in 0.5% neopeptone, the serum therefore contains 0.32 unit streptomycin per ml.

Calculation:

$$\frac{\text{Streptomycin inhibiting test organism}}{\text{Amount serum inhibiting test}}$$

(a) in 0.5% neopeptone $\frac{0.16}{0.5} = 0.32$ unit streptomycin/ml.

(b) in 0.25% neopeptone $\frac{0.032}{0.5} = 0.064$ unit streptomycin/ml.

Should the serum have been diluted before processing the test, the result must necessarily be multiplied by the dilution factor.

Summary. 1. A simple, easily readable method for the clinical determination of streptomycin in body fluids has been described, requiring a minimum amount of test fluid.

2. *Klebsiella pneumoniae No. 41* which grows luxuriantly in simple media and is readily inhibited by streptomycin was adopted as test organism.

3. The assay was made more sensitive by utilizing relatively deficient media which made the test organisms more susceptible to the action of streptomycin.

4. It is possible, because *Klebsiella pneumoniae No. 41* is insensitive to penicillin, to perform streptomycin level determinations in patients who are receiving streptomycin and

penicillin treatment concomitantly.

5. By the use of this method it has been noted that streptomycin is retained in the blood in effective therapeutic amounts for relatively longer periods of time, thereby permitting more convenient spacing of injections. The clinical application of these studies will be made the subject of a subsequent report.

15573 P

Cocarboxylase and the Synthesis of Acetylcholine.

Bruno Minz. (Introduced by Robert Chambers.)
From the Laboratory of General Physiology, Paris University.

Biochemical studies of nerves under electrical stimulation or at rest have shown that acetylcholine, regarded for a long time only as a transmitter of nervous impulses at synaptic levels, is present in appreciable amounts in the nerve fiber itself.[1] We have proved that extracts from stimulated nerves produce a more intense action on the dorsal muscle of the leech than extracts from nerves at rest. This finding led us to consider the part played by acetylcholine in the phenomenon of nerve conduction. An analysis of the observed effects enabled us to reach the conclusion that excitation is followed not only by an increase in the amount of acetylcholine in nerves but also by the formation of an unknown substance appearing in the system and capable of sensitizing the reactive muscle. Further work has made clear analogies of action between this unknown substance and thiamin and has enabled us to prove that stimulated nerve liberates an amount of thiamin many times greater than that given off by the nerve at rest.[1] Since the amount of thiamin which sensitizes the muscle of the leech to acetylcholine is much smaller than that which would inactivate cholinesterase, we concluded that the vitamin plays a part in the synthesis of the chemical transmitter.

Thiamin is active in organisms in the form of thiamin pyrophosphate or cocarboxylase. Therefore, it seemed desirable to determine whether this coenzyme may play a part in the synthesis of acetylcholine from choline.

A contracture of the dorsal muscle of the leech may be obtained with relatively high doses of choline. But contrary to the reaction produced by acetylcholine, this effect is not increased by eserine.[2,3] If cocarboxylase is capable of transforming choline into acetylcholine, the mixture of both should exert an action on the muscle which is increased by eserine. Experiments have shown that cocarboxylase alone is without any effect on the leech muscle preparation whether treated with eserine or not. The coenzyme, however, increases the action on the muscle of a contracture-producing concentration of choline in the absence of eserine. This increase is even greater after the muscle has been treated with eserine. With threshold doses of choline, at which cocarboxylase has no visible effect, eserine has brought about a clear reinforcement. Thus, the muscle may aerobically and in the presence of cocarboxylase transform choline into an ester the action of which is increased by eserine. These are the biological properties of acetylcholine.

How may these facts be interpreted? We know that cocarboxylase intervenes at a particularly important level in the metabolism of carbohydrates. It catalyzes the decarboxylation of pyruvic acid so that acetic acid is produced. From the work of Banga, Ochoa and Peters[4,5] it also appears that this decarboxylation is associated with phosphorylation of adenylic acid to yield adenosine triphosphate. Nachmansohn and Machado[6] have shown that adenosine triphosphate intensifies the activity of an enzyme system (choline acetylase) which produces the final synthesis of the cholinergic transmitter. Our knowledge about this enzyme is still rudimentary, but if the enzyme should prove specific, the disintegration of pyruvic acid initiated by cocarboxylase should furnish the system with 2 important elements, *viz.*, acetic acid and adenosine triphosphate, concerned with the synthesis of acetylcholine from choline.

---

[1] Binet, L. A., and Minz, B., *C. R. Soc. de Biol.*, 1934, **115**, 1169; **116**, 107; *Arch. internat. physiol.*, 1936, **42**, 281; Minz, B., and Agid, R., *C. R. Arch. Soc.*, 1937, **205**, 576; Minz, B., *C. R. Soc. de Biol.*, 1938, **127**, 1251; 1939, **131**, 1156.

[2] Fühner, H., *Arch. f. exp. Path.*, 1918, **82**, 51.

[3] Minz, B., *Arch. f. exp. Path.*, 1932, **168**, 292.

[4] Banga, I., Ochoa, S., and Peters, R. A., *Biochem. J.*, 1939, **33**, 1980.

[5] Ochoa, S., *J. Biol. Chem.*, 1941, **138**, 751.

---

[6] Nachmansohn, D., and Machado, A. L., *J. Neurophysiol.*, 1943, **6**, 397.

15574

Effect of Pemphigus Serum on the Behavior of Rats.

David I. Macht and Marion C. Insley.

From the Department of Pharmacology, Laboratories of the Sinai Hospital, Baltimore.

Macht and Pels,[1] by employing special phytopharmacological methods have demonstrated the presence of a toxic substance in the blood of pemphigus patients. This toxic reaction is not given by other dermatoses so that the phytopharmacological test has been found useful in establishing the diagnosis of pemphigus at an early stage and in doubtful cases.[2-4] More recently, Macht and Ostro have extended our knowledge concerning this substance and have employed the test in evaluating therapeutic procedures employed in treating this disease.[5] From these experiments it was evident that pemphigus is not a simple dermatosis but is primarily a constitutional or systemic disease or a form of toxemia. Concomitant with the characteristic skin eruptions the patient exhibits profound disturbances of a systemic nature, the most prominent symptoms of which come from the nervous system. This has been noted already 50 years ago by Payne[6] who, in his classical lecture on pemphigus, stated:

"If any system of the body is affected besides the skin it seems to be the nervous system rather than any other. Obscure symptoms of nervous depression are sometimes recognized before the eruption begins, and in the severer forms the cerebral nervous system—or at least the brain—is evidently profoundly affected, as is shown by delirium and nervous prostration."

In order to investigate further the effects of this toxic substance in pemphigus serum on the nervous system the present writer undertook experiments on the behavior of white rats by 3 kinds of experimental psychological methods. First, a study was made on the behavior of the rats in Watson's circular maze. While views differ as to the complete significance of the process through which the rats undergo the learning of the maze problem, it is generally agreed that such a performance involves not only a response to the proprio-sensitive stimuli but also a *choice* reaction or faculty of *discrimination,* which the rats exhibit under the urge of hunger. A second series of experiments was made on rats which have been trained to walk a tightly stretched rope 8 feet long from one platform to another on which food had been placed. This performance involves a delicate neuro-muscular coordination. In the third series of experiments, rats were trained to climb a tightly stretched per-

---

[1] Macht, D. I., and Pels, I. R., Proc. Soc. Exp. Biol. and Med., 1927, **25**, 237.

[2] Pels, I. R., and Macht, D. I., *Arch. Dermat. and Syph.*, 1929, **19**, 640.

[3] Pels, I. R., and Macht, D. I., *Arch. Dermat. and Syph.*, 1931, **23**, 601.

[4] Pels, I. R., and Macht, D. I., *Arch. Dermat. and Syph.*, 1934, **29**, 206.

[5] Macht, D. I., and Ostro, M., *Urologic and Cutaneous Rev.*, 1946, **19**, 271.

[6] Payne, J. F., *Lancet*, 1893, **2**, 421.

TABLE I.

Influence of Sera on Behavior of Rats.

Exp. No.	Wt of rat g	Method of inj.	Kind of serum inj.	Dose cc	Phyto-toxic index %	Behavior before inj.			Behavior 30 min. after inj.			Behavior 2 hr later			Remarks
						In maze sec.	On rope sec.	Climbing sec.	In maze	On rope sec.	Climbing sec.	In maze	On rope sec.	Climbing sec.	
I	250	Periton.	*Pemph. vulg.*	0.5	55	6.4			Depressed stalled			Completely stalled			Marked depression
II	"	Subcut.	" "	0.5	54	12.0 1 error			Completely stalled			Completely stalled			Marked dep.
III	"	Periton.	" "*	0.5	43	7.8			Stalled depressed			Marked depress.			Dies overnight
IV	"	"	" *foli.*	0.5	45	7.5			Marked depress.			Depressed stalled			" late same day
V	150	Subcut.	" "	0.3	45	9.0	3.5	4.3	9.1 sec.	3.5	5.5	20.3 sec.	6.1	8.7	Dep. min. dose
VI	"	"	" *vulg.*	1.0	56	7.6	4.9	11.2	10.8 "	4.5	13.2	10.0 "	4.7	13.1	Dep.
VII	"	Periton.	" "	1.0	43	6.1	3.3	8.0	10.4 "	4.7	15.3	11.8 "	6.2	10.0	"
VIII	"	"	" "	0.5	52	7.7	5.6	9.5	35.7 " 2 errors	5.5	11.9	10.2 " 1 error	5.2	15.5	Marked dep.
IX	"	Subcut.	" "	0.6	52	7.7	6.2	12.6	10.5 sec.	8.3	10.0	8.0 sec.	5.4	10.0	Dep.
X	"	"	" *foli.*	1.0	59	13.1 6 errors	5.0	7.0	24.9 " 8 errors	4.8	7.7	25.6 " 5 errors	6.3	9.9	Great dep. next day
XI	"	Periton.	" "	1.0	59	6.2	3.9	5.7	Prostrated	Pros.	Pros.	Pros.	Pros.	Pros.	Dies overnight
XII	"	Subcut.	" *vulg.*	1.0	55	7.3	4.1	9.8	10.5 sec.	6.8	19.1	13.3 sec.	Stalled	21.7	Marked dep.
XIII	"	Periton.	" " during remission	1.0	60	6.3	3.6	6.2	8.1 "	4.9	9.0	6.0 "	Depressed 4.5	8.0	Mild dep. during remission period
XIV	"	Subcut.	*Pemph. foli.*	0.3	58	12.5 2 errors	4.1	5.2	10.0 " 1 error	3.9	8.1	17.3 " 2 errors	4.3	7.9	Mild dep. from minute dose
XV	"	"	" "	0.4	58	6.2	4.3	12.8	6.4 sec.	4.3	10.5	9.0 sec.	5.0	11.6	Mild dep.
A	250	Periton.	Normal human	0.5	72	7.5			7.9 "			7.2 "			Control sera
B	"	"	" "	0.5	70	6.7			6.4 "			6.1 "			" "
C	"	Subcut.	" "	1.0	75	5.8			6.1 "			5.4 "			" "
D	"	Periton.	Saline	4.0	100	6.8			6.9 "			6.4 "			Physiol. saline
E	150	"	Pernicious anemia	0.5	52	8.9	4.9	4.9	7.7 "	4.5	5.8	8.3 "	3.4	4.1	Control pern. anemia
F	"	Subcut.	Normal human	0.5	70	5.1	3.7	7.5	5.2 "	3.9	7.7	5.1 "	3.7	8.0	Control
G	"	"	" "	1.0	70	6.0	5.1	14.2	6.3 "	5.1	14.7	6.0 "	5.4	14.4	"
H	"	"	" "	0.5	72	5.1	3.7	5.0	4.9 "	2.7	3.3	4.9 "	3.3	5.2	"
I	"	"	" "	0.5	73	5.1 2 errors	3.2	4.3	8.2 " 2 errors	3.2	4.1	5.6 " 2 errors	3.3	4.3	"

---

* Mouth

pendicular rope. This exercise also involves neuro-muscular coordination but is especially designed for testing the work capacity of the skeletal muscles. A detailed description of the methods employed is given by the senior author elsewhere.[7]

Fifteen experiments were performed with injections of small amounts of pemphigus serum into trained rats and an equal number of experiments were performed with normal and other control sera. For obvious reasons it was not advisable to employ the same rats too frequently because of the danger of anaphylactic reaction. In results reported here no allergic reactions were elicited. Two groups of white rats were employed. One group consisted of large-sized male rats each weighing approximately 250 g. The other and larger group consisted of younger white male rats each weighing approximately 150 g. The larger animals were trained only in the circular maze whereas the smaller and lighter rats were trained by all the 3 methods described above. The injections were made in some cases intraperitoneally while subcutaneous injections were made in other cases. Table I gives the results obtained in the 15 experiments with pemphigus serum and also in 9 control experiments. It will be noted that in one of the controls an injection of physiological saline alone was given, while in another control experiment, an injection was given of the blood serum from a case of pernicious anemia which, as was shown by one of the authors, is toxic for living plants.[8] Pernicious anemia blood, however, is not depressant for rats. Table I gives the weight of the rats, the method of injection, the dose of serum injected, the phytotoxic index of the sera and the performance of the animals before injection and after injection. In every case the running time in the maze is an average of 3 runs and when the animals made mistakes by running into blind alleys the number of such errors is noted. In the experiments on rope walking and rope climbing the time in seconds is given also as an average of 3 runs on the horizontal rope and 2 climbs on the vertical rope.

By the phytotoxic index in Table I is meant the ratio of the growth of the seedling roots in 1% serum solution to its growth in the normal control solution according to the formula,

$$\text{Phytotoxic index of growth} = \frac{X}{N} \times 100$$

in which N represents the average growth of controls and X the average growth in the 1% serum solution.

Results. It was noted that in the control experiments neither the injections of physiological saline nor of normal blood serum produced much depression or any other effect on the behavior of the rats. On the other hand, pemphigus sera produced a definite depression and in some cases a very marked prostration. This effect was noted not only in the maze experiments which indicate the influence of the toxic constituent on the "intelligence" of the animals but also on the neuro-muscular coordination as indicated by the running on the horizontal rope and especially in a weakening of the muscles as revealed in the climbing experiments. Two kinds of pemphigus sera were examined. In the majority of the experiments, serum from cases of pemphigus vulgaris was injected, but in several of the other experiments the serum from 2 cases of pemphigus foliaceus was injected. Two rats, one large one and one small one, died after injections of the latter variety.

The toxic effects obtained in this investigation throw a light on the profound constitutional symptoms of patients afflicted with pemphigus and stress the fact that the cutaneous and mucous membrane lesions are but the external manifestations of a grave systemic disturbance which so far as is known is not a bacterial infection but which is definitely characteristic of a toxemia, as has been proven by pharmacological experiments. It has been claimed that in the late stages of pemphigus there is an increased amount of organic phosphorus and a reduced calcium content of the blood. The senior author, in many previous experiments with such blood sera and with sera from cases of inorganic acidosis, did not obtain such depressant effects on the central nervous system after such small doses of serum, as were obtained in the present research. In the opinion of the authors, pemphigus should be described in treatises on internal diseases with equal propriety as smallpox, leprosy, syphilis, pellagra, and other constitutional diseases are discussed in such text books.

---

[7] Macht, D. I., *Exp. Med. and Surg.*, 1943, **1**, 260.

[8] Macht, D. I., *J. Pharm. and Exp. Therap.*, 1926, **29**, 461.

Summary. 1. The neuro-muscular responses and general behavior of white rats were studied, (1) in a circular maze, (2) on animals trained to walk a tightly-stretched horizontal rope, and (3) on animals trained to climb a vertically-stretched rope.

2. Injections of small quantities of pemphigus sera in these rats produced a definite depression of performance in all 3 tests, while control experiments with normal sera did not affect the rats adversely.

3. These findings corroborate the authors' experiments with pemphigus sera on plants and indicate that this disease is a systemic, rather than a cutaneous one, as is indicated by the toxic effects exerted by the blood serum of patients with pemphigus.

15575

The Vitamin B_6 Group. IX. Comparative Growth and Anti-anemic Potencies of Pyridoxal, Pyridoxamine and Pyridoxine for Dogs.*

P. S. SARMA, E. E. SNELL, AND C. A. ELVEHJEM.

From the Department of Biochemistry, College of Agriculture, University of Wisconsin, Madison.

Fouts *et al.*[1,2] and subsequently others[3,4] showed that dogs, when kept on vitamin B_6 deficient diets, developed a characteristic microcytic hypochromic anemia which could be cured with pyridoxine. Anemia has also been produced in chicks,[5] ducks[6] and swine[7] by feeding diets low in vitamin B_6.

Since enzymatically effective derivatives of vitamin B_6 are related directly to pyridoxal[8,9] and pyridoxamine,[10] rather than to pyridoxine, and since the 3 compounds have equivalent growth-promoting activities when injected intraperitoneally into rats or chicks,[11] it seemed possible that the antianemic potencies of the former 2 compounds might surpass that of pyridoxine. Results reported below show that when injected intravenously, pyridoxal, pyridoxamine and pyridoxine are equally active in increasing hemoglobin levels and supporting growth of dogs.

Experimental. Littermate male pups 8 weeks old (No. 41 and 42) and one older female pup 14 weeks old (No. 40) were treated twice with an anthelmintic (tetrachlorethylene), freed of external parasites with rotenone and placed in individual cages. The vitamin B_6-deficient diet had the following

---

* Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. This work was supported in part by grants from the Wander Company, Chicago, Ill. We are indebted to Merck and Company, Inc., Rahway, N.J., for the generous supply of crystalline B vitamins and to the Abbott Laboratories, North Chicago, Ill., for the generous supply of haliver oil.

For paper VIII of this series see reference 11.

[1] Fouts, P. J., Helmer, O. M., Lepkovsky, S., and Jukes, T. H., *J. Nutrition*, 1938, **16**, 197.

[2] Fouts, P. J., Helmer, O. M., and Lepkovsky, S., *J. Nutrition*, 1940, **19**, 393.

[3] Borson, H. J., and Mettier, S. R., PROC. SOC. EXP. BIOL. AND MED., 1940, **43**, 429.

[4] McKibbin, J. M., Schaefer, A. E., Frost, D. V., and Elvehjem, C. A., *J. Biol. Chem.*, 1942, **142**, 77.

[5] Luckey, T. D., Briggs, G. M., Jr., Elvehjem, C. A., and Hart, E. B., PROC. SOC. EXP. BIOL. AND MED., 1945, **58**, 340.

[6] Hegsted, D. M., and Rao, M. N., *J. Nutrition*, 1945, **30**, 367.

[7] Cartwright, G. E., Wintrobe, M. M., and Humphreys, S., *J. Biol. Chem.*, 1944, **153**, 171.

[8] Bellamy, W. D., Umbreit, W. W., and Gunsalus, I. C., *J. Biol. Chem.*, 1945, **160**, 461.

[9] Green, D. E., Leloir, L. F., and Nocito, V., *J. Biol. Chem.*, 1945, **161**, 559.

[10] Umbreit, W. W., O'Kane, D. J., and Gunsalus, I. C., *J. Bact.*, 1946, **51**, 576.

[11] Sarma, P. S., Snell, E. E., and Elvehjem, C. A., *J. Biol. Chem.*, 1946, **165**, 55.

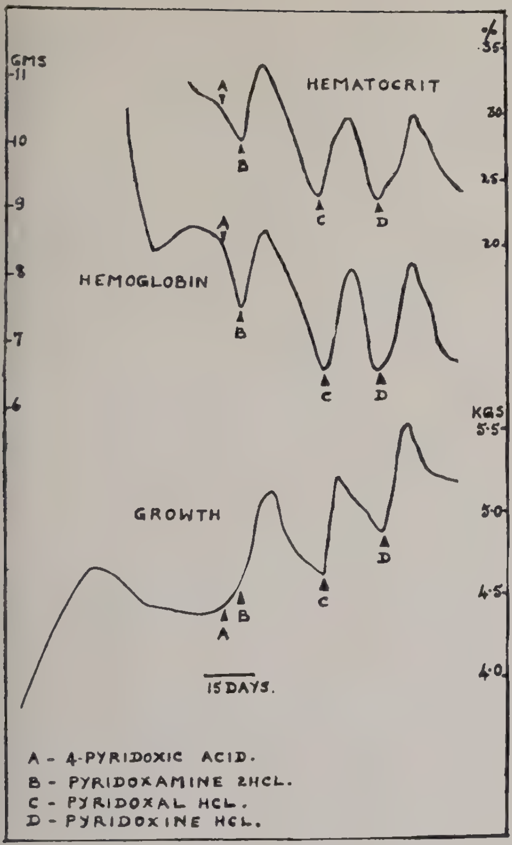


Fig. 1.
Growth, hemoglobin and hematocrit curves of Dog No. 40.

composition: sucrose 66, acid-washed alcohol extracted casein 19, cotton seed oil 11, salts IV[12] 4. All animals received food and water *ad libitum.* The animals also received an aqueous suspension of all the B vitamins except vitamin B_6, twice a week, the administration being based on the following daily requirement of the vitamins per kg body weight of the dog: thiamin 0.1 mg, riboflavin 0.1 mg, calcium pantothenate 0.5 mg, nicotinic acid 2.0 mg, choline 50 mg and biotin[13] 0.025 mg. Halibut liver oil was given twice a week to supply 1800 I.U. of vitamin A and 500 I.U. of vitamin D per dog per day. The dogs were weighed semiweekly during the depletion period and more frequently when the actual experiments were in progress. Hemoglobin determinations[14] and hematocrit readings were carried out on 3 ml samples of blood obtained from the radial vein of the dog before the morning feeding. These determinations were made twice weekly during the depletion period, but more often during the period of supplementation.

Results. The results obtained with dog No. 40 are shown in Fig. 1. After an initial increase, the rate of growth showed a plateau and then decreased slowly. After 2 months, the hemoglobin level of this dog reached a low figure of 8.48 g per 100 ml of blood. At this point (A) daily injections of 4-pyridoxic acid (40 μg per kg of body weight) were made into the radial vein for 4 consecutive days (total dose 704 μg). A slight

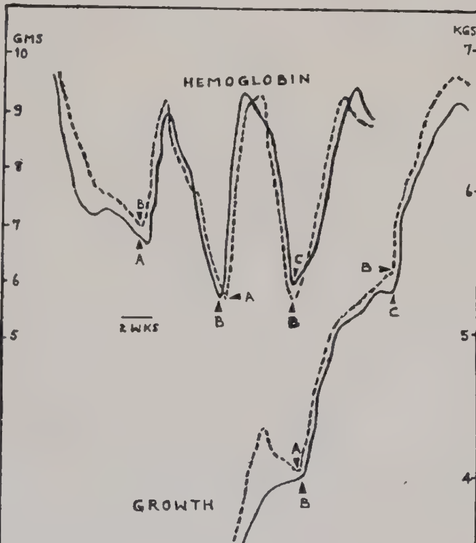


Fig. 2.
Growth and hemoglobin curves of Dogs No. 41 and 42.

---

[12] Phillips, P. H., and Hart, E. B., *J. Biol. Chem.*, 1935, **109**, 657.

[13] Ruegamer, W. R., Michaud, L., Elvehjem, C. A., and Hart, E. B., *Am. J. Physiol.*, 1945, **145**, 23.

[14] Evelyn, K. A., *J. Biol. Chem.*, 1936, **115**, 63.

gain in body weight accompanied by a marked decrease in hemoglobin and hematocrit readings occurred. At point B, pyridoxamine dihydrochloride was similarly injected. There was a prompt response in growth and in hemoglobin and hematocrit levels. A total of 860 μg of pyridoxamine dihydrochloride was injected over a period of 4 days. This amount was insufficient to raise the blood level to the maximum value. Subsequently, the dog soon began to lose weight and again became anemic. Following this second depletion period a very similar response resulted from the administration of an equimolar quantity of pyridoxal hydrochloride (point C) and finally from pyridoxine hydrochloride (point D). It is apparent from Fig. 1 that the 3 members of the vitamin B_6 group are equally active in promoting growth and blood regeneration.

These results were confirmed and extended in experiments with dogs No. 41 and 42 (Fig. 2). These 8-week-old pups showed lower hemoglobin and hematocrit values than the older dog No. 40, after they were kept for a period of 2 months on the vitamin B_6-deficient diet described earlier. Dog No. 41 received pyridoxine hydrochloride by injection at 40 μg/kg of body weight, while dog No. 42 was injected with an equimolar amount of pyridoxal hydrochloride. These injections were given daily for 5 consecutive days. Increases in weight and hemoglobin were approximately the same in both dogs. In contrast to the findings with the older dog No. 40, which, though heavier, received a relatively smaller amount of vitamin B_6, cessation of vitamin injections did not result in a rapid decrease in weight, though the hemoglobin values decreased in a short time. After 3 weeks, when the hemoglobin values were sufficiently reduced, dog No. 41 received pyridoxal hydrochloride while dog No. 42 received pyridoxine hydrochloride at the same level for a period of 7 days. This interchange in the administration of pyridoxine and pyridoxal was effected to eliminate possible biological variations in response of the 2 animals. Again there was a prompt response which was greater than in the previous case due to the longer period of administration. The weight gains as well as the increases in blood hemoglobin and hematocrit values were almost identical in both cases. As a result of the increased amounts of pyridoxine and pyridoxal which were injected, the dogs gained weight even after vitamin administration was discontinued. However, the hemoglobin values decreased gradually and this decrease was aided by the withdrawal of 100 ml of blood from the saphenous artery in both dogs. After 3 weeks, dog No. 41 was given pyridoxamine dihydrochloride while the other dog was given pyridoxal hydrochloride. Supplementation was at the same level (40 μg/kg of body weight) as in the preceding case and was again continued 7 days. Again, a prompt response in growth and hemoglobin values resulted (Fig. 2) and the responses were of equal magnitude in the 2 animals. In all these cases, the hematocrit values changed in a manner similar to the hemoglobin values.

Discussion. Pyridoxal, pyridoxamine and pyridoxine, when injected in equimolar amounts, are equally active in promoting growth in dogs. These results agree with earlier observations made on the growth-promoting property of these compounds in vitamin B_6-deficient rats and chicks.[11] When examined by their ability to promote blood regeneration in anemic dogs, the 3 compounds likewise appear to be equally active. Separate experiments, which it seems unnecessary to report in detail, showed that the antianemic potencies of the 3 compounds for chicks were likewise equal when the compounds were administered by injection. By analogy with the results obtained in rats and chicks,[11] administration of pyridoxal and pyridoxamine mixed in the ration of the dogs should decrease their apparent antianemic potency as compared with that of pyridoxine. This had not been tested experimentally. 4-Pyridoxic acid is inactive in promoting growth and blood regeneration in the vitamin B_6-deficient dog. It has also been found inactive for rats.[15]

Summary. Pyridoxal, pyridoxamine and pyridoxine were administered by intravenous injection to vitamin B_6-deficient dogs. The 3 compounds were equally active in promoting growth and blood regeneration in this species.

---

[15] Snell, E. E., and Rannefeld, A. N., *J. Biol. Chem.*, 1945, **157**, 475.

15576

Effect of Certain Anti-anemic Substances upon Serum Cholinesterase Activity.*

JOHN EMERSON DAVIS.

From the Pharmacological Laboratory, University of Arkansas School of Medicine, Little Rock.

It has been shown that hyperchromic anemia can be produced in dogs by the daily administration of choline or acetylcholine, and that this anemia is abolished by the use of liver extract or pteroyl glutamic acid (folic acid).[1,2] It was further shown that an acetylcholine-like activity present in the serum of anemic dogs after the feeding of choline, was abolished or diminished after the onset of treatment with the antianemic substances. Certain evidence was presented to show that both liver extract and pteroyl glutamic acid probably acted by increasing the cholinesterase activity of the body.

The present article is concerned with a study of the effects of liver extract and folic acid in increasing the serum cholinesterase activity both *in vitro* and *in vivo*.

Procedure and Results. Serum cholinesterase activity was determined by the electrometric titration at a constant pH of 7.38, of the acetic acid liberated from a solution of 100 mg of acetylcholine bromide in 25 cc of water by 1 cc of blood serum in 10 minutes. The method is essentially that of Goodman, Carlson, and Gilman,[3] except that titrations were made at room temperature (28-31°C) and to a pH of 7.38. A Leeds and Northrup pH meter with a glass electrode was employed. Cholinesterase activity was estimated in terms of cc of 0.01 N sodium hydroxide required to neutralize the acetic acid liberated over a period of 10 minutes.

The *in vitro* experiments consisted of the incubation of 1 cc of dog's blood serum with various injectable liver extracts or with pteroyl glutamic acid† for 2½ hours at 37°C. It was necessary to add a little disodium phosphate to render the pteroyl glutamic acid soluble in water. The volume of solution employed was usually kept at 0.1 cc and maximal effect was usually obtained with 0.15 mg of folic acid per cc of serum. When liver extracts were used in the incubation tests, it was found impractical to use more than 0.1 unit (U.S.P.) of the crude liver extracts (*i.e.*, 1 or 2 units per cc), because the use of greater amounts caused inhibition of the cholinesterase in 1 cc of serum. The concentrated liver extracts (15 units per cc) were usually diluted 1 in 10 with distilled water and used in amounts rarely exceeding 0.3 U.S.P. unit, as this quantity seemed to give maximal effect if it gave any with a particular serum. Some dog sera do not show an increase of cholinesterase activity when incubated with either one or the other of these substances. We found, among our dogs, one whose serum showed a marked increase of the enzyme activity when incubated with either folic acid or liver injection. We created others by the experimental injection of di-isopropylfluorophosphate (D.F.P.)‡ which has been shown to inhibit irreversibly, or perhaps destroy cholinesterase.[4-6]

Fig. 1 shows the effects of incubation of 1 cc of serum with stated amounts of anti-anemic material upon its cholinesterase activity in terms of per cent increase. The

---

* Research paper No. 828, Journal Series, University of Arkansas.

[1] Davis, J. E., *Science*, 1946, **104**, 37.

[2] Davis, J. E., *Am. J. Physiol.*, 1946, **147**, 404.

[3] Goodman, Carlson, and Gilman, *J. Pharmacol. and Exp. Therap.*, 1939, **66**, 15.

† Synthetic *L. casei* factor (Folvite) was kindly furnished by Lederle Laboratories, New York, New York.

‡ Furnished by the Medical Division of the Chemical Warfare Service, Edgewood Arsenal, Md., through the courtesy of Col. John R. Wood.

[4] Bodansky, O., and Mazur, A., *Fed. Proc.*, 1946, **5**, 123.

[5] Comroe, J. H., Todd, J., Koelle, G. B., and Gilman, A., *Fed. Proc.*, 1946, **5**, 172.

[6] Mazur, A., and Bodansky, O., *J. Biol. Chem.*, 1946, **163**, 261.

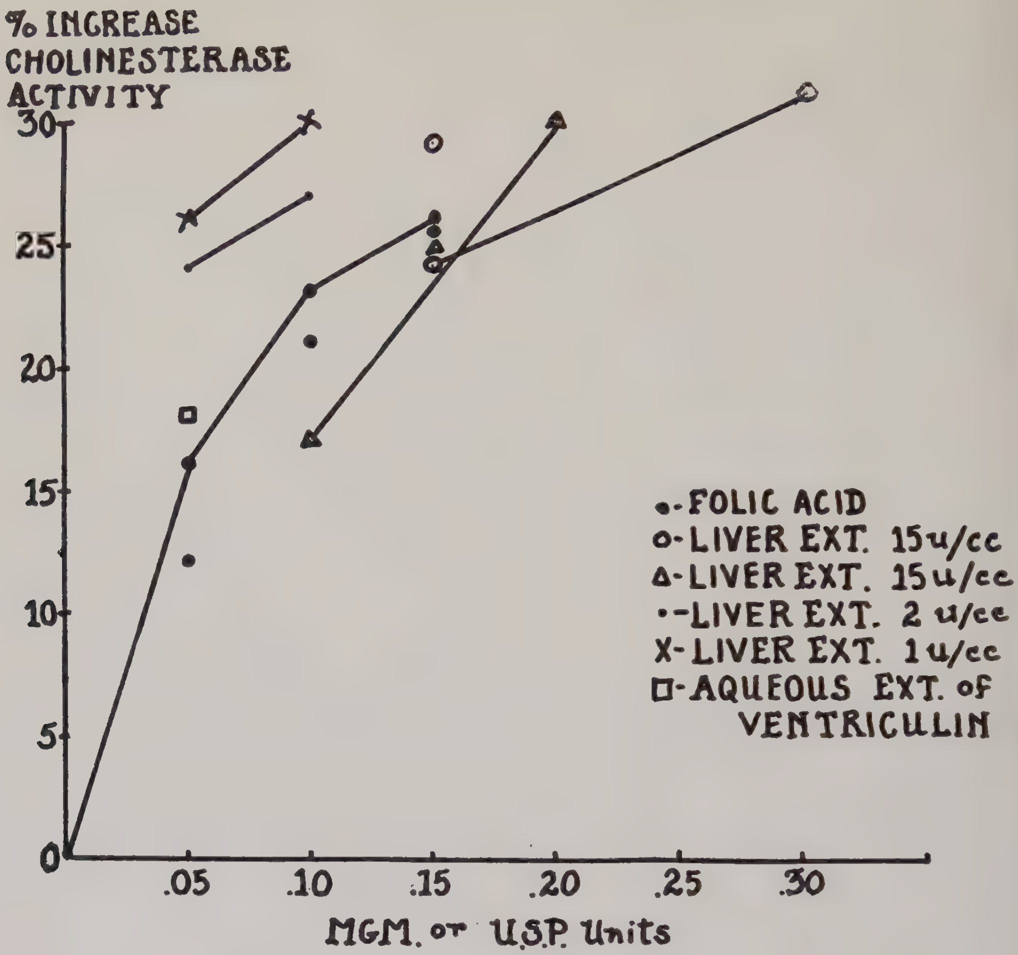


Fig. 1.

The effect of incubation of various amounts of antianemic preparations with dog serum upon its cholinesterase activity.

Amounts of folic acid used are given in terms of mg, while the amounts of other substances are given in terms of U.S.P. units according to their labelled potencies. All points connected by intact lines were obtained upon one sample of serum from one dog. All other points represent experiments on one sample of serum obtained from an animal treated with D.F.P. 2 days earlier.

data here shown were collected on the sera of 2 dogs that showed approximately the same capacity for increase of serum enzyme activity. All points connected by lines were obtained on tests of the serum of one dog obtained from one sample of blood. This dog had been untreated for 2 months prior to this experiment, but had previously received injections of acetylcholine twice daily for 2 months. All points not connected by lines, represent experiments made upon one serum sample from another dog which had received an injection of 1 mg of D.F.P. 2 days before the tests. It will be seen in Fig. 1 that the maximal increase in enzyme activity that was achieved under the conditions of this experiment was about 30%. The concentrated (high potency) liver extracts appear to be less effective (per U.S.P. unit as labelled) than the crude liver extracts investigated. Similar experiments have been performed on serum from several other dogs, but the sera used in the tests shown in Fig. 1 seem to be the most suitable for comparison of folic acid with different liver extracts. On the basis of other experiments as well as

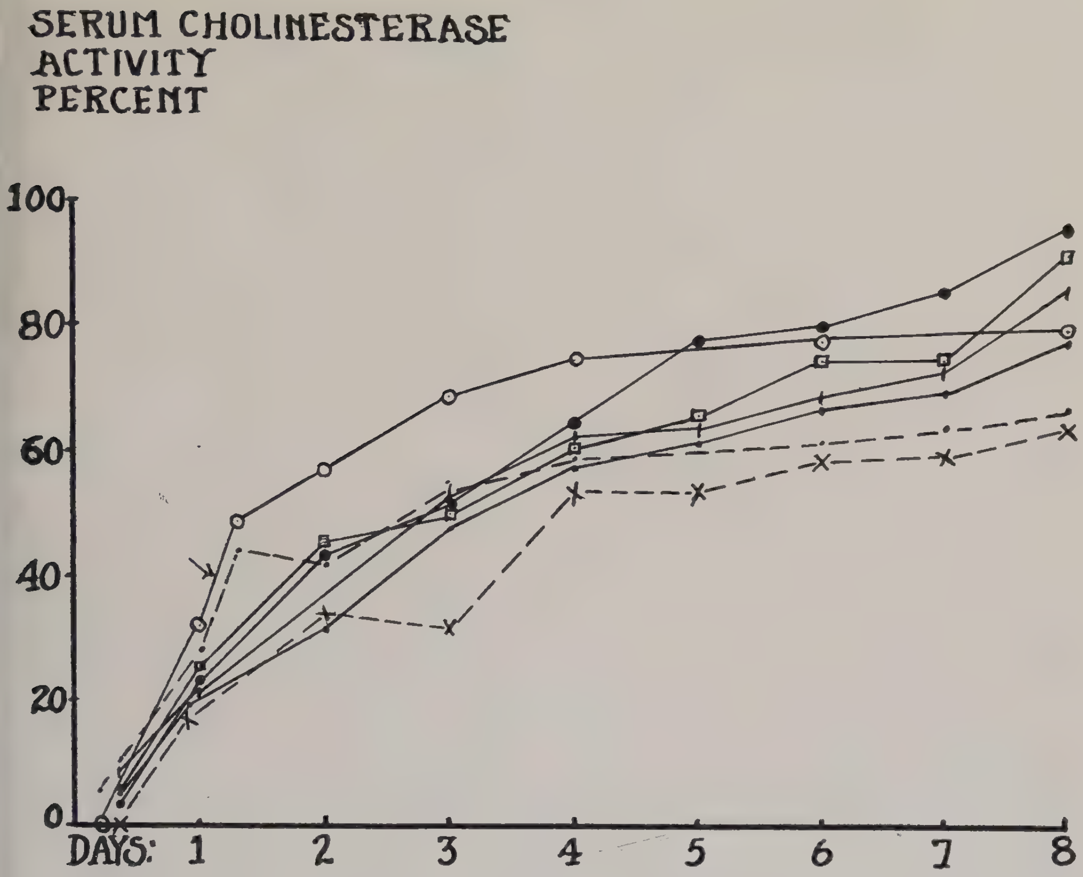


Fig. 2.

The effect of liver extract and pteroyl glutamic acid administration upon the regeneration of serum cholinesterase activity in dogs given D.F.P.

D.F.P. (diisopropyl-fluorophosphate) was injected intramuscularly into all dogs at zero time.

Liver extract and folic acid were administered from the 2nd to 5th days to all dogs represented by solid intact lines, except one (open circles) which received 2.4 mg of folic acid at the arrow.

these, it seems that 1 mg of pteroyl glutamic acid is roughly equal to 0.5 unit of the crude extracts or 1.5 units of the concentrated liver extracts used, in increasing cholinesterase activity. An aqueous extract of stomach U.S.P. (Ventriculin, Fig. 1) also appeared to be effective in increasing the activity of this enzyme in the serum.

The *in vivo* experiments consisted of injecting dogs with diisopropyl-fluorophosphate (D.F.P.), and studying the subsequent rate of regeneration of the cholinesterase activity of the serum. Fig. 2 shows the effect of the i.m. injection of 1 mg of D.F.P. upon the serum cholinesterase levels of 6 dogs. A 7th dog represented by open dots was given 0.6 mg (.05 mg per kg) because his body weight was markedly less than that of the others. It will be seen in Fig. 2 that the D.F.P. caused rapid and great reductions of the serum cholinesterase activities to rates ranging from 0 to 8% of the preexperimental levels. Beginning on the first day after the injections of D.F.P. 4 of the dogs were treated intensively with liver extracts and folic acid for 5 days, while 2 dogs (dashed lines) served as untreated control dogs. The other dog (open dots, Fig. 2) received only one injection of 2.4 mg of folic acid at 27 hours after the D.F.P. administration. It will be noted in Fig. 2 that the 5 treated dogs all showed higher serum cholinesterase activities after the 5th day following D.F.P., than the 2 untreated control animals. The differences in serum cholinesterase prevailing between treated and untreated animals on the 8th day after D.F.P. ranged from 11 to 32%.

Discussion. The D.F.P. was administered intramuscularly in aqueous solution to 2 of the animals (open dots and top dashed line, Fig. 2). We found by experience that the D.F.P. lost potency rapidly when diluted in water, and for this reason we diluted our next sample in olive oil and injected the remainder of the animals intramuscularly with the oil solution. The 2 dogs injected with aqueous solution of D.F.P. showed recovery of their serum cholinesterase activities to normal in 15 and 21 days (for treated and untreated animals, respectively) but the other animals were not observed beyond the 8th day. From the 2nd to 4th days the smallest dog, which had received the low dose of 0.6 mg of D.F.P. showed the greatest regeneration of enzyme activity. It therefore seemed wise to give the other dogs a single dose of 1 mg irrespective of body weight. Variation in body weights do not account for any differences in rapidity of cholinesterase regeneration, as far as we can ascertain.

Liver extract or folic acid alone were given to particular animals at the start of the experiment shown in Fig. 2. Since no rapid changes were produced thereby, we commenced giving both preparations to all experimental dogs, except one, on the 3rd experimental day. The average amounts given each dog were 2 mg daily of folic acid (pteroyl glutamic acid) and 5 units of liver extract.

If proof should come forth that the cholinesterase potentiating actions of liver and folic acid are the important actions of these substances in the treatment of certain human anemias, then it would seem likely that liver preparations could be assayed by one of the methods reported in this paper.

Summary and Conclusions. The incubation of liver extracts or pteroyl glutamic acid with certain dog blood sera caused an increase in the cholinesterase activity of the sera. From such experiments it was estimated that 1 mg of pteroyl glutamic acid was roughly equal to 1 ± 0.5 U.S.P. unit of the liver extracts examined, in cholinesterase activity potentiating power.

The administration of liver extracts and pteroyl glutamic acid to dogs in which the serum cholinesterase had been greatly depleted by diisopropyl-fluorophosphate injections, caused more rapid regeneration of this enzyme activity than was observed in untreated control animals.

15577

Effect of Riboflavin and Other B Complex Vitamins on Work of Perfused Frog Muscles.

N. W. Shock* and W. H. Sebrell.

From the Division of Physiology, National Institute of Health, United States Public Health Service, Bethesda, Md., and the Baltimore City Hospitals, Baltimore, Md.

Previous experiments have shown that when appropriate concentrations of thiamine,[1,2] cocarboxylase,[2] pantothenic acid,[3] or pyridoxine,[4] were added to the perfusion fluid; the work output of the frog gastrocnemius muscles was significantly increased. The fol-

---

* With the technical assistance of Frances S. Berg.

[1] Shock, N. W., and Sebrell, W. H., *Am. J. Physiol.*, 1944, **142**, 265.

[2] Shock, N. W., and Sebrell, W. H., Proc. Soc. Exp. Biol. and Med., 1945, **59**, 212.

[3] Shock, N. W., and Sebrell, W. H., *Am. J. Physiol.*, 1944, **142**, 274.

[4] Shock, N. W., and Sebrell, W. H., *Am. J. Physiol.*, 1946, **146**, 399.

TABLE I.
Effect of Riboflavin on Total Work Output (Averages of 10 Animals for Each Concentration).

	Avg work done				
Conc. mM/L	Control leg g-cm	Exp. leg g-cm	Mn diff.* exp.-control leg g-cm	C.R.†	% incr.
.0000	1697	1829	132 ± 70.3	1.9	7.8
.0000001	1716	1790	74 ± 95.3	0.8	4.3
.0000005	1916	2003	87 ± 110.8	0.8	4.6
.000001	1999	2202	203 ± 76.3	2.7	10.1
.000005	1516	1790	274 ± 140.0	2.0	18.1
.00001	2097	2380	283 ± 82.9	3.4	13.5
.0001	2163	2395	232 ± 58.4	4.0	10.7
.0005	1993	2245	252 ± 31.9	7.9	12.6
.001	1864	2179	315 ± 84.4	3.7	16.9
.005	1896	2257	361 ± 73.9	4.9	19.0
.01	2085	2281	196 ± 62.3	3.1	9.4
.05	1811	1945	133 ± 111.0	1.2	7.4
.1	1583	1727	144 ± 92.6	1.6	9.1

* Mean difference ± 1 standard error of the mean.

† Critical ratio $= \dfrac{\text{Mean difference}}{\sigma_{\text{Mn. diff.}}}$

lowing report presents the results of similar tests made with the addition of riboflavin alone, as well as the simultaneous addition of all the vitamins of the B complex previously tested.

Experimental. Fatigue curves were recorded graphically for each gastrocnemius muscle of the frog under the same conditions of perfusion, loading, stimulation, and recording as previously described.[1,3] After recording the fatigue curve on the first gastrocnemius muscle, riboflavin was added in known concentrations to the buffered glucose-Ringer's solution used for perfusion of the frog while the fatigue curve was recorded on the second gastrocnemius muscle. Ten animals were tested under each experimental condition. Individual work curves for the control and vitamin-treated muscles were plotted for each animal, and the total work done was estimated from the areas under the curves as measured with a planimeter. Standard errors of all average values were calculated using formulae for small numbers.

Results. Effects of Increasing Concentrations of Riboflavin. The average total work output of control and experimental muscles exposed to increasing concentrations of riboflavin in the perfusion fluid is shown in Table I. A statistically significant increase in work output was observed when the concentration of riboflavin in the perfusion fluid was maintained between 0.00001 and 0.01 mM per liter. Concentrations of riboflavin greater or less than these limits failed to show a significant improvement in work output.

Effect of Increasing Concentrations of Riboflavin on the Distribution of Work Output. The average height of contraction at each minute of the work period was calculated for the control and riboflavin-treated muscles. The average difference between the control and vitamin-treated muscles at each minute was calculated in millimeters and also as the percentage of the height of contraction of the control muscle at that time. From these calculations, the maximum absolute differences and the maximum percentage differences shown for each riboflavin concentration were tested for statistical significance as shown in Table II. From this table, it may be seen that the average height of contraction of the vitamin-treated muscles is significantly greater than that of the untreated muscles after 9-17 minutes of exercise when the perfusion fluid contains riboflavin in contractions of 0.000005 to 0.01 mM per liter. At riboflavin concentrations of 0.05 or 0.1 mM per liter, no significant effect was observed.

TABLE II.
Effect of Riboflavin on Extent of Contraction of Perfused Frog Muscles.

	Avg height of contraction of muscle mean difference (exp.-control leg) (Mean of 10 animals at each concentration)							
	At max. absolute difference				At max. percentage* difference			
Ribo-flavin conc. mM/L	Min. of exercise	Mean absolute diff.†	C.R.‡	% diff.§	Min. of exercise	Mean absolute diff.†	C.R.‡	% diff.§
.0000000	10	1.0 ± 0.8	1.3	3.0	13	0.8 ± 0.6	1.3	3.0
.0000001	9	1.0 ± 0.4	2.5	5.6	16	0.5 ± 0.4	1.0	5.0
.0000005	9	4.3 ± 2.9	1.5	17.7	12	2.8 ± 1.8	1.6	20.3
.000001	10	8.0 ± 2.0	4.0	25.9	17	4.5 ± 1.5	3.0	43.3
.000005	9	11.0 ± 4.1	2.7	32.1	16	5.1 ± 1.8	2.8	54.7
.00001	8	12.3 ± 2.1	5.9	45.3	11	7.1 ± 1.8	3.8	57.9
.0001	9	8.2 ± 1.5	5.3	39.4	13	5.0 ± 1.2	4.1	59.4
.0005	11	10.0 ± 1.4	7.1	48.3	18	6.8 ± 1.1	6.2	60.0
.001	8	10.2 ± 1.9	5.4	42.8	13	6.1 ± 1.1	5.5	73.0
.005	10	12.1 ± 2.3	5.3	55.0	15	6.9 ± 1.3	5.3	82.1
.01	11	7.7 ± 1.6	4.8	46.4	17	4.4 ± 1.1	4.0	71.0
.05	17	6.0 ± 2.0	3.0	32.1	12	5.1 ± 1.8	2.8	38.0
.1	11	5.1 ± 2.9	1.8	39.8	14	3.9 ± 1.7	2.3	51.3

* Calculated as $\dfrac{\text{Ht. of experimental curve–Ht. control curve}}{\text{Ht. of control curve}} \times 100.$

† Mean ± 1 standard error of the mean.

‡ $\text{C.R.} = \dfrac{\text{Mean difference}}{\sigma_{\text{Mn. diff.}}}$

§ Calculated as

$$\frac{\text{ht. of experimental curve — ht. of control curve}}{\text{ht. of control curve}} \times 100$$

TABLE III.
Effect of Mixture of Vitamins of the B Complex on Total Work Output of Perfused Frog Muscles.

	Avg work done				
Exp. conditions	Control leg g-cm	Exp. leg g-cm	Mn. diff. Exp.-Control leg g cm	C.R.	% incr.
Vit. B mixture*	1811	2201	390 ± 75.2	5.2	21.5
Ringer's + 20 units insulin per L.	1663	1681	18 ± 70.6	0.2	1.1
Vit. B mixture* + 20 units insulin per L.	1639	2022	383 ± 62.5	4.1	23.3

* See text for composition of vitamin mixture.

Effect of Simultaneous Administration of Vitamins of the B Complex on Total Work Output. Experiments were performed in which the second leg of the frog was perfused with buffered glucose-Ringer's solution to which the following vitamins of the B complex were added simultaneously: 0.005 mM thiamine hydrochloride per liter, 0.01 mM calcium pantothenate per liter, 0.1 mM nicotinic acid amide per liter, 0.0001 mM pyridoxine hydrochloride per liter, and 0.0005 mM riboflavin per liter. The first line of Table III shows that the work output was significantly increased by the addition of vitamins to the perfusion fluid.

Discussion. The experiments with riboflavin show that there is an optimum concentration at which improvement in total work output of perfused frog muscles is observed. For riboflavin, this optimum concentration is 0.0005 mM per liter. As shown before for thiamine hydrochloride, calcium pantothenate and pyridoxine hydrochloride, at increased concentrations of the vitamin, the improvement in work output is no longer significant.

The experiments with the mixture of vitamins of the B complex give no evidence that the improvement in work output observed with the vitamins individually is additive, since the degree of improvement observed with the mixture was of the same order of magnitude as that observed with the individual vitamins. Thus, if it is assumed that the improvement in work output observed with the addition of vitamins to the perfusion fluid results from metabolic changes, there must be some other substance or substances essential to the recovery process which impose a ceiling on the energy available for muscle contraction. Such substances might conceivably be oxygen or glucose. Although ample glucose was present in the perfusion fluid, it seemed possible that the frog was unable to supply sufficient insulin for its metabolic use. Hence, additional experiments were performed to test this hypothesis. Control experiments were first run, in which 20 units of insulin were added to each liter of perfusion fluid used for the second leg. The second line of Table III shows that no significant improvement in work output resulted. This finding is in accordance with similar tests made by Eddy.[5] Insulin (20 units per liter) was then added to the vitamin mixture which was used in perfusing the second leg. The results shown in line 3 of Table III give no support to the hypothesis that utilization of glucose is the limiting factor in determining the improvement in work output following the addition of vitamins of the B complex to the perfusion fluid, since the improvement was no greater than that observed without the addition of excess insulin.

Summary. The work output of the gastrocnemius muscle of the perfused frog has been determined following the addition of riboflavin to the perfusion fluid in concentrations ranging from 0.0000001 to 0.1 mM per liter.

The total work output was significantly increased by the addition of 0.00001 mM riboflavin per liter. Concentrations below this level were ineffective. A significant improvement in total work output was observed with riboflavin concentrations up to and including 0.01 mM per liter; with concentrations of 0.05 and 0.1 mM per liter, the improvement was not statistically significant.

The extent of muscle contraction after 8-11 minutes of work was significantly increased by the addition of 0.00001 to 0.01 mM of riboflavin per liter of perfusion fluid.

When a mixture of thiamine hydrochloride, calcium pantothenate, nicotinic acid amide, pyridoxine hydrochloride and riboflavin, each at optimal concentrations is added to the perfusion fluid; the improvement in total work output is no greater than that observed with the most effective vitamins singly (thiamine hydrochloride, calcium pantothenate, and pyridoxine hydrochloride).

The addition of 20 units of insulin per liter of perfusion fluid along with the vitamin mixture did not result in any greater increase in work output than that observed with the vitamins alone.

---

[5] Eddy, N. B., *Am. J. Physiol.*, 1924, **69**, 432.

15578

A New Endpoint for Blood Pressure Determination in the Rat's Tail.*

LEONARD T. SKEGGS, JR., AND JACK R. LEONARDS. (Introduced by V. C. Myers.)

From the Department of Clinical Biochemistry, School of Medicine, Western Reserve University, Cleveland, Ohio.

The indirect plethysmographic method for the measurement of systolic blood pressure in the caudal artery of the rat by the method of Byrom and Wilson,[1] as adapted by Williams, Harrison, and Grollman,[2] and also by other workers,[3] has found widespread application. Among the difficulties encountered in the use of this method is the occasional appearance of equivocal endpoints. We wish to describe a device which may be used in place of the plethysmograph and which we believe gives more reliable endpoints in trained rats.

In the plethysmographic method a change in volume of the tail occurs when the pressure in an inflated cuff at the base of the tail is allowed to drop to the arterial pressure. This small change in volume of the tail is conducted through a thin rubber membrane to a rigid water system and thence to a capillary tube where it is noted as a rise in the water level, and is regarded as the endpoint for the determination. In the device which we have constructed, this same change in volume of the tail is mechanically transferred to develop a pressure on a carbon telephone transmitter button. This increased pressure on the button results in an approximate 75% decrease in its electrical resistance which may be observed on a continuously-indicating ohmmeter circuit.

Mechanically, the device (Fig. 1) consists of an H-shaped figure formed of balsa wood, the upright on the left being hinged at its middle to one end of the crosspiece, the other end of the crosspiece being rigidly fixed to the righthand upright. Thus a lever is formed whose action is similar to a nasal speculum. At the upper end of the lever and in a plane perpendicular to it, are secured flat plates of light sheet metal. The inner edges of these plates are straight, 5.5 cm long, and parallel. A cloth band is glued between the upper surfaces of these plates and allows a 1.5 cm separation of their inner edges. At the lower end of the H-shaped lever is secured a carbon telephone transmitter button, the flat grounded portion of the button being placed against the righthand upright. At the lower end of the lefthand upright, and mounted on the lower part of the hinge is an adjusting screw. This screw is so placed that it may be adjusted to touch the center, ungrounded, diaphragm portion of the button at any degree of separation of the upper plates. The overall height of the device is 7 cm, the fulcrum of the hinge is 4 cm from the upper surface of the plates, and the uprights are separated by the 2.5 cm crosspiece.

Electrical connections are made to the grounded and to the ungrounded portion of the button with the most flexible wire that is consistent with strength. The wires may be soldered directly to the button. The wire from the ungrounded, diaphragm portion is mechanically secured to the righthand upright to prevent transmission of wire movements to this sensitive portion of the button.

The electrical leads from the device may be connected to a simple ohmmeter circuit capable of indicating 0-300 ohms continuously without electrical adjustments. A wide dial instrument is obviously desirable. We prefer, however, to use a simple wheatstone bridge circuit, the first 2 arms of which are formed by a 400-ohm radio type potentiometer, the third arm by a 200-ohm fixed carbon

---

* We will be pleased to loan, for a short period of time, a sample device to any interested individuals.

Supported by a grant from the Smith, Kline, and French Company, Philadelphia, Pa.

[1] Byrom, F. B., and Wilson, C., *J. Physiol.*, 1938, **93**, 301.

[2] Williams, J. R., Jr., Harrison, T. R., and Grollman, A., *J. Clin. Invest.*, 1939, **18**, 373.

[3] Kemph, G. F., and Page, I. H., *J. Lab. Clin. Med.*, 1942, **27**, 1192.

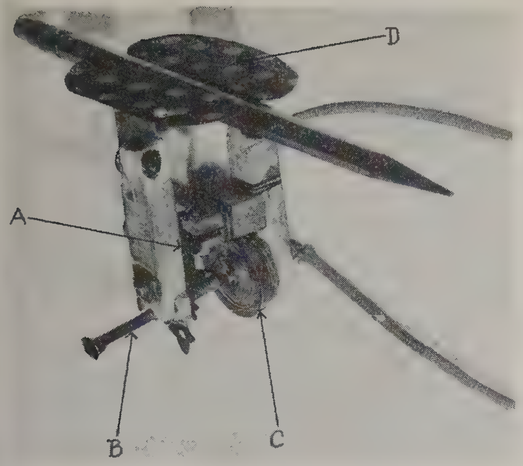


Fig. 1.

Photograph of endpoint device for indirect blood pressure measurement in the rat's tail. "A" is a hinge. "B" is an adjusting screw touching diaphragm portion of carbon button "C." "D" is one of 2 metal plates covered and joined with a cloth band. The pencil shows the position occupied by the rat's tail.

resistance, and the fourth arm by the device itself. As a balance indicator we use a 0-150 microammeter, and as a source of current, a 1.5-volt dry cell in series with a 5000-ohm variable resistance. In this circuit the setting of the variable resistance controls the sensitivity, while the setting of the potentiometer controls the working range. It is not necessary at any time to "balance" the bridge accurately since only changes in the resistance of the button are desired and not the actual resistance.

In order to take blood pressure readings the tail of the rat is led through a 20 mm pressure cuff[4] and thence into a tube fashioned of wire screen which simply supports the tail in a horizontal position. A gap of approximately 6.5 cm is left between the cuff and the tail holder. The endpoint device is clamped to the tail in this gap by forming the cloth band like a sling around the tail from below and setting the adjusting screw up against the center of the carbon button so that the parallel edges of the metal plates approach on the upper side of the tail. The pressure with which the device is clamped to the tail is entirely noncritical with respect to obtaining endpoints and need only be sufficient that there be no danger of its becoming detached. The carbon button proper is then tapped lightly which loosens the carbon particles and increases their resistance to approximately 200 to 300 ohms. The pressure in the cuff is then rapidly elevated to a point above the expected blood pressure of the animal and slowly lowered. At the endpoint, at which the arterial pressure fills the tail with blood, the indicator of the ohmmeter will commence a characteristic smooth movement in the direction of decreasing resistance. If further fall in cuff pressure be prevented at this point the movement will continue until a resistance value of 20 to 50 ohms is obtained. Should the cuff pressure be allowed to fall without interruption this characteristic movement of the ohmmeter indicator will occur much more rapidly. When this low resistance value has been obtained one may, in a sense, confirm his reading by allowing the cuff pressure to fall rapidly below the venous pressure of the rat and observing the return of the ohmmeter indicator to its former value. Undue movements of the rat will result in sharp, jerky, nonpersistent movements of ohmmeter indicator. There is no difficulty in differentiating these movements from the true endpoint.

It has been shown by Sobin[5] that the accuracy of the indirect plethysmographic method of blood pressure measurement may be greatly increased by local warming of the tail. We have accordingly mounted the pressure cuff, endpoint device and tail holder in a warm air chamber controlled thermostatically to 43°C ± 1°C. The rat's body is enclosed in a wire screen holder on the outside of the chamber. We have found that after placing a rat in this apparatus, at least 5 to 15 minutes are necessary in order to secure a maximum reading. Better results are obtained if the entire rat is first warmed at 40-42°C for 10 minutes according to the method of Williams, Harrison and Grollman.[2] However, in this case, as shown by Proskauer *et al.*[6] one must accept the possibility of a systemic error due to elevation of the rats' body temperature.

---

[4] Schuler, R. H., Kupperman, H. S., and Hamilton, W. F., *Am. J. Physiol.*, 1944, **141**, 625.

[5] Sobin, S. S., *Am. J. Physiol.*, 1946, **146**, 179.

In order that we might compare the 2 methods we have replaced the tail holder with a simple plethysmograph and have simultaneously recorded cuff pressures by both carbon button and plethysmographic endpoints. Over a large number of trials we have found general agreement within 5 to 15 mm, the carbon button endpoint nearly always being the higher. Often the readings were identical.

Discussion. The method described may be used on either anesthetized or unanesthetized animals. If anesthesia is not objectionable a very satisfactory endpoint may be more simply obtained by inserting 26-gauge syringe needles about 3 cm apart into the tail distal to the pressure cuff. These needles serve as electrodes and are connected to a sensitive direct current wheatstone bridge. Thus the actual resistance of the rat's tail is measured and is found to decrease when blood flows into it.

Summary. A new endpoint for the indirect measurement of systolic blood pressure in the caudal artery of the rat has been described. The change in volume of the rat's tail which occurs when the pressure in an inflated cuff at the base of the tail is allowed to drop to arterial pressure is mechanically transferred to develop pressure on a carbon telephone transmitter button. The electrical resistance of the button is continuously observed, a decrease in resistance indicating the endpoint for the blood pressure measurement.

---

[6] Proskauer, G. G., Neumann, C., and Graef, I., *Am. J. Physiol.*, 1945, **143**, 290.

15579

Chemotherapeutic Action of Various Forms of Penicillin on Hemolytic Streptococcal Infections in Mice.*

GLADYS L. HOBBY, BLANCHE BURKHART, AND BEVERLY HYMAN.
From the Biological Laboratories, Chas. Pfizer & Co., Broooklyn, N.Y.

The existence of at least 4 distinct forms of penicillin was first revealed in 1945.[1,2] The various penicillins differ widely in biological activity as evidenced by the fact that their potency varies from approximately 900 to 2300 Oxford units per mg when assayed *in vitro* against a standard strain of *Staphylococcus aureus*. Differences in the biological activity of these penicillins *in vivo* have also been reported recently by Eagle and Musselman[3] and by Coghill, Osterberg, and Hazel.[4] The penicillin in clinical use during the past 4 years has contained varying proportions of these penicillins as well as varying amounts of unidentified impurities.[5] It has been suggested by Dunham and Rake[6] and by Lewis[7] that the impurities in commercial penicillin may play a role in the total efficacy of the drug *in vivo*.

In 1943 it was observed by one of us

---

* This work was presented at a Penicillin Conference held under the auspices of the U. S. Public Health Service, Food and Drug Administration, and National Research Council, Washington, D.C., on March 26-27, 1946.

[1] Dale, Sir Henry, *Science*, 1945, **101**, 23.

[2] Report by the Committee on Medical Research, O.S.R.D., Washington, and the Medical Research Council, London, on Chemistry of Penicillin, *Science*, 1945, **102**, 627.

[3] Eagle, H., and Musselman, A., *Science*, 1946, **103**, 618.

[4] Cogill, R. D., Osterberg, A. E., and Hazel, G. R., *Science*, 1946, **103**, 709.

[5] Coghill, R. D., *Chemical and Engineering News*, 1945, **23**, 2310.

[6] Dunham, W. B., and Rake, G., *Am. J. Syph.*, 1945, **29**, 214.

[7] Lewis, M. R., *Science*, 1944, **100**, 314.

(G.L.H.)[8] that a total of 60 Oxford units of preparations of crude or commercial penicillin gave partial protection against hemolytic streptococcal infections in white mice, whereas a total of 100 Oxford units afforded complete protection to 90 to 95% of the animals. In similar experiments in 1944 it was observed that the administration of 3 times as much crystalline penicillin G (*i.e.*, 300 units) was necessary to obtain the same degree of protection in comparably infected mice. Subsequent comparisons of certain of these preparations which were originally tested in 1943 with other preparations of crystalline penicillin G showed similar significant differences in therapeutic efficacy. Accordingly, studies were undertaken to determine the relative value of the various forms of purified penicillin and of "commercial" penicillin.

Crystalline penicillin G and preparations containing predominantly or only penicillin X, F, and K were studied as well as random samples of material manufactured for general clinical use.

Preliminary observations on the bacterial spectrum of these preparations gave scant information. Whereas certain differences could be detected by the *Bacillus subtilis-Staphylococcus aureus* differential ratio[†] as determined by the Oxford cup plate method,[9] this technic was not practicable with most pathogenic organisms. Only with certain strains, such as *Eberthella typhosus* which appeared to be more sensitive to penicillin X than to the other penicillins, was it possible to demonstrate a difference in any of the forms of penicillin. Using a modified dilution technic, differences in the sensitivity of various species of microorganisms to penicillin may be easily demonstrated.[10-12] However differences in the sensitivity of an individual organism to the different forms of penicillin were not sufficiently great to be demonstrable by this procedure. As it appeared that the relative value of the various forms of penicillin could not be satisfactorily determined *in vitro*, the following studies *in vivo* were conducted.

Hemolytic streptococcus, strain $C_{203}Mv$, was used as the infecting organism throughout the study. One cc of 10^{-4}, 10^{-5}, 10^{-6} and 10^{-7} dilutions of a 14-hour rabbit's blood broth culture was injected intraperitoneally into each of a series of 20 g white mice. A minimum of 6 to 10 mice was used for each dilution of each series. Treatment was started 1 to 2 hours after the infecting dose and the penicillin was administered subcutaneously in peanut oil[13] in divided dosage. Forty per cent of the total dose was administered 1 to 2 hours, 40% 7 to 8 hours, and 20% 24 hours after infection. Total dosages of 15, 30, 60, 100, 150, 210, 250, 300, 500 and 600 units were used. A control series of untreated animals was always included in each day's experiment. The untreated infection was always uniformly fatal within 18 to 48 hours. The results may be seen in Tables I, II, and III.

Purified Penicillins.[‡] Crystalline penicillin G afforded little or no protection in total dosages of 30, 60, or 100 units. With 150 or 210 units, there was partial protection

---

[8] Hobby, G. L., and Dawson, M. H., unpublished data.

[†] Hereinafter designated differential ratio. The differential ratios of crystalline penicillin X, G, F, and K are accepted as 1.2-2.0, 1.0, 0.66, and 0.38 respectively. The differential ratio does not indicate the penicillins present in mixtures. In the absence of penicillin X, a ratio of 1.0 probably indicates penicillin G only whereas a ratio below 1.0 indicates the presence of more than one form.

[9] Schmidt, W. H., Ward, G. E., and Coghill, R. D., *J. Bact.*, 1945, **49**, 411.

[10] Chain, E., *et al.*, *Lancet*, 1940, **2**, 226.

[11] Hobby, G. L., Meyer, K., and Chaffee, E., PROC. SOC. EXP. BIOL. AND MED., 1942, **50**, 277.

[12] Keefer, C. S., and Anderson, D. G., *Penicillin in the Treatment of Infections*, Oxford Medical Publications, Oxford University Press, 1945.

[13] Hobby, G. L., Meyer, K., and Chaffee, E., PROC. SOC. EXP. BIOL. AND MED., 1942, **50**, 285.

[‡] With the exception of penicillin X, all purified or crystalline preparations of penicillin were obtained through the kindness of Dr. R. Pasternack, Dr. E. V. Brown, and Dr. P. Regna of the Research Laboratory of Chas. Pfizer and Co., Brooklyn, N.Y.

We are indebted to Dr. Henry Welch of the Food and Drug Administration for the sample of crude penicillin X and to Lederle Laboratories, Inc., for the sample of crystalline penicillin X.

TABLE I.
Chemotherapeutic Action of Crystalline and Purified Penicillins on Hemolytic Streptococcal Infections (Group A) in Mice.

Form of penicillin		X	X	G	F	K	Controls
Oxford units per mg		1069	170	1667	960	2017	
Bc. subtilis / *Staph. aureus*		1.39	1.16	1.0	0.66	0.38	
Dilution of culture*	Total dosage in units	Therapeutic effect: % survival					
10^{-4}	30	40	40	25			3
-5		30	70	0			1
-6		60	80	0			6
-7		70	80	50			10
10^{-4}	60	87.5†	80†	32			
-5		100	90	22			
-6		100	90	66			
-7		100	100	66			
10^{-4}	100	87.5	70	60	57		
-5		100	100	54	80		
-6		100	100	69	57		
-7		100	100	90	100		
10^{-4}	150			30	20		
-5				80	60		
-6				70	10		
-7				86	90		
10^{-4}	210	100		80			
-5		100		70			
-6		100		90			
-7		100		90			
10^{-4}	300	100		90†	80†	40	
-5		100		100	80	50	
-6		100		100	90	80	
-7		100		100	100	80	
10^{-4}	500					100†	
-5						80	
-6						90	
-7						100	

* A minimum of 6 to 10 mice were used for each dilution in each set. One cc of a 10^{-7} dilution contained 1-10 lethal doses of hemolytic streptococci; 10^{-6}, 10-100; 10^{-5}, 100-1000; and 10^{-4}, 1000-10,000.

† Curative dose_{90} = Level at which 90% of infected animals survived.

and with 300 units almost complete protection. The preparation of penicillin G used had a potency of 1667 o.u./mg and a differential ratio of 1.0.

Crystalline penicillin X (potency 1069 o.u./mg, differential ratio 1.27 to 1.39) gave partial protection with only 30 units and almost complete protection with 60 units. Thus a comparable degree of protection was supplied by 60 units of penicillin X and 300 units of crystalline penicillin G.

Essentially the same results were obtained with a low potency preparation thought to contain only penicillin X. This preparation had a potency of 170 o.u./mg and a differential ratio of 1.16. The action was apparently not influenced by the presence of a high concentration of impurities.

A high potency preparation of penicillin K (potency of 2017 o.u./mg, differential ratio 0.38), which contained relatively little impurity, gave appreciably less protection than either penicillin G or X. A total dosage of 300 units of K was required to afford protection comparable to that supplied by 100 to 150 units of penicillin G or 30 units of penicillin X. A larger dosage, equivalent to 500 units of penicillin K, gave almost complete protection. The protective action of 500 units of penicillin K was equivalent to that of 300 units of crystalline penicillin G or 60 units of crystalline penicillin X.

Only partially purified preparations of penicillin F were available. Three hundred units of a preparation containing 960 o.u./mg (differential ratio 0.66) gave approximately

TABLE II.
Chemotherapeutic Action of Commercial Preparations of Penicillin on Hemolytic Streptococcal Infections (Group A) in Mice.

Preparation of penicillin‡		I	II	III	IV	V	VI	VII	Controls
Oxford units per mg		281	685	955	1004	1071	1057	1175	
$\frac{\textit{Bc. subtilis}}{\textit{Staph. aureus}}$		1.0	0.58	0.80				0.65	
Dilution of culture*	Total dosage in units	Therapeutic effect: % survival							
10^{-4}	30		20	50					3
$^{-5}$			10	44					1
$^{-6}$			40	27					6
$^{-7}$			70	72					10
10^{-4}	60	50	60		100†		75	90†	
$^{-5}$		80	90	67	100	17	75	80	
$^{-6}$		90	80	84	100	100	100	90	
$^{-7}$		100	100	67	100	100	100	100	
10^{-4}	100	100†	90†		100		100†	100	
$^{-5}$		100	100	84†	100	67†	100	100	
$^{-6}$		80	100	84	100	100	100	100	
$^{-7}$		100	100	100	100	100	100	100	
10^{-4}	150	100	100				100	100	
$^{-5}$		100	100				100	100	
$^{-6}$		80	100				100	100	
$^{-7}$		100	100				100	100	

* A minimum of 6 to 10 mice were used for each dilution in each set. One cc of a 10^{-7} dilution contained 1-10 lethal doses of hemolytic streptococci; 10^{-6}, 10-100; 10^{-5}, 100-1000; and 10^{-4}, 1000-10,000.

†Curative dose$_{90}$ = Level at which 90% of infected animals survived.

‡ Preparations I and V were prepared from *Penicillium notatum,* strain 832; preparations IV and VI from *Penicillium chrysogenum,* strain 1951B25; preparations II and III from *Penicillium chrysogenum,* strain X-1612; and preparation VII from *Penicillium chrysogenum,* strain Q176.

TABLE III.
A Comparison of the Chemotherapeutic Action of Various Forms of Penicillin in Hemolytic Streptococcal Infections in Mice.

Penicillin	Curative dose$_{90}$ Units	Curative dose$_{90}$ μg*	Relative activities Biological	Relative activities Gravimetric
Penicillin X	60	67	500	270
” G	300	180	100	100
” F	slightly >300	>195	<100	<92
” K	500	220	60	82
Prep. I (crude)†	100	60	300	300
Preps. II-VII (crude)	60-100		300-500	

* Based on potencies of 900, 1667, 1550, and 2300 units per mg for X, G, F, and K respectively.

† $\frac{\textit{Bc. subtilis}}{\textit{Staph. aureus}}$ differential ratio on Prep. I = 1.0; gravimetric values calculated on basis of pure penicillin G at 1667 Oxford units per mg.

the same degree of protection as 300 units of crystalline penicillin G.

On a unitage basis, the relative chemotherapeutic efficacy of penicillins X, G, F and K in hemolytic streptococcal infections in mice was on the order of 500, 100, slightly less than 100, and 60 respectively.¶ Converting to a gravimetric basis, the relative order of their efficacy was 270, 100, slightly less than 92, and 82 respectively[§] (Table I and III).

¶ Preliminary experiments carried out after this manuscript was submitted for publication indicate that, on a unitage basis, the relative efficacy of penicillin dihydro F to crystalline penicillin G is approximately 143 to 100.

Commercial Penicillin.[‖] Preparations of "commercial" penicillin were chosen at random from material manufactured between 1943 and 1946. Samples of penicillin produced by all of the 4 major strains of Penicillium used in production during that time were included. The potency of these preparations varied from 281 o.u./mg to 1175 o.u./mg. Likewise the differential ratio of these preparations varied from 0.58 to 1.0. In all cases the penicillin was recovered from the fermentation liquor by adsorption onto activated carbon, elution with organic solvents, and subsequent extraction into chloroform or chlorinated hydrocarbons. This procedure would presumably eliminate penicillin X.

Preparation I was among those originally tested in 1943. On retesting one and again 2 years later, partial protection was obtained with only 60 units of this penicillin, whereas 100 units gave almost complete protection. One hundred units of preparation I was as effective as 300 units of crystalline penicillin G, tested simultaneously with the same dilutions of the same culture on the same days. The results were constant. It was after this observation had been confirmed that the differential ratio of preparation I was determined and found to be 1.0—the same as the value for crystalline penicillin G. In the absence of penicillin X this information suggested that probably only penicillin G was present in preparation I.

Six other preparations of penicillin manufactured during 1944, 1945, and 1946 gave comparable results as indicated in Table II. In general 60 to 100 units of crude penicillin was as effective as 300 units of crystalline G. Crude penicillin with a potency of less than 300 o.u./mg was as effective against hemolytic streptococcal infections in mice as material with a potency of 1000 o.u./mg and vice versa. Likewise, crude preparations with a low differential ratio were as effective as those with a high ratio.

Preparation VII, having a low differential and known to contain a high percentage of penicillin K in addition to other penicillins, gave complete protection with fewer units than was necessary for a comparable degree of protection when either crystalline penicillin G or highly purified K were used. Crystalline penicillin G, highly purified penicillin K, and preparation VII were tested on the same day, using the same experimental conditions throughout. Although relatively pure penicillin K was less effective than crystalline penicillin G, the presence of penicillin K in the crude or commercial preparation did not alter its protective action against this particular experimental infection.

It appears that crude or commercial preparations contain a substance which enhances the action of the penicillins present. All of the crude preparations tested were more effective against hemolytic streptococcal infections in mice than crystalline penicillin G or purified preparations of penicillin F or K. Although the crude preparations were all more effective than penicillin K, they were no more effective than crystalline penicillin X. (Tables II and III).

Summary. The comparative efficacy of 4 forms of penicillin and random samples of "commercial" penicillin has been studied in experimentally-produced hemolytic streptococcal infections in mice. On a unitage basis the relative chemotherapeutic efficacy of penicillin X, G, F and K under the experimental conditions used, was on the order of 500, 100, slightly less than 100, and 60 respectively. On a gravimetric basis, the relative order of efficacy was 270 for X, 100 for G, slightly less than 92 for F and 82 for K. The values for penicillin F were obtained with a preparation which contained impurities, the action of which is not known.

[§] These values were based on CD_{90} levels. On a CD_{50} basis the relative order of efficacy of penicillin X, G, F, and K was gravimetrically 127, 100, 57, and 40 respectively. Eagle and Musselman[3] have recently reported on this basis values of 260, 100, 50, and 9. The relative order is the same in the two series. The difference in the actual values reported by Eagle and Musselman and by ourselves may be due at least in part to the vehicle used for administration of the penicillin.

[‖] All commercial penicillins used in these studies were prepared by Chas. Pfizer & Co., Brooklyn, N.Y.

All of the samples of "commercial" penicillin were 3 to 5 times as effective as crystalline penicillin G in protecting the animals against the infection. Moreover, the protective action exerted by these impure penicillins was of the same order regardless of the unitage per mg or the value of the *Bacillus subtilis-Staphylococcus aureus* differential ratio. The protective power of pure X is as great as that observed following the administration of the impure "commercial" penicillins. As the latter were manufactured by a process which presumably excludes the presence of X, it would not seem that the superiority of the impure penicillins over crystalline G could be attributed to the presence of X in the impure material.

15580 P

Nicotinic Acid and Pantothenic Acid Content of Sunflower Seed Meal and Some Oil Seed Products.*

AVIS RECTOR, HARRY G. DAY, AND L. S. MCCLUNG.

From the Laboratories of Chemistry and of Bacteriology, Indiana University, Bloomington, Ind.

Interest in the nutritional significance of sunflower seed meal has been aroused by recent reports, based on feeding experiments, that it is a good source of B complex vitamins[1] and protein.[2,3] Such procedures indicated that the content of pantothenic acid is relatively lower than most of the other B complex vitamins. Also, chemical analyses for nicotinic acid in this product gave uncertain results. Therefore, in this investigation particular attention was given to the determination of these 2 vitamins by microbiological methods. Several products in addition to sunflower seed meal were analyzed.

Well known analytical methods for pantothenic acid[4,5] and nicotinic acid[6] were used.

The materials assayed were commercial products. All, except one, had been defatted by solvent extraction processes.

The analytical results, as shown in Table I, reveal that sunflower seed meal contains approximately 150% more preformed nicotinic acid than does the best grade of wheat germ analyzed, and it is approximately 500% richer in this vitamin than is corn germ or solvent-extracted soybean meal. Of the materials analyzed, only sunflower seed meal showed the presence of the "alkali-labile precursor" of nicotinic acid.[7] Since the precursor is biologically active in both dogs and chicks,[7] the total nicotinic acid value for sunflower seed meal is approximately the same, if not higher, than that of peanut meal.[8] This is of considerable interest because the latter has been rated as an outstanding source of nicotinic acid, as compared with other edible oil seed and cereal products.[9]

The pantothenic acid content of the sunflower seed meal is similar to that of the solvent-extracted soybean meal but it is ap-

---

* Aided by a grant from the VioBin Corporation.

[1] Day, H. G., and Levin, E., *Science*, 1945, **101**, 438.

[2] Mitchell, H. H., Hamilton, T. S., and Beadles, J. R., *J. Nutrition*, 1945, **29**, 13.

[3] Grau, C. R., and Almquist, H. J., PROC. SOC. EXP. BIOL. AND MED., 1945, **60**, 373.

[4] Skeggs, H. R., and Wright, L. D., *J. Biol. Chem.*, 1944, **156**, 21.

[5] Cheldelin, V. H., and Williams, R. R., *Ind. and Eng. Chem., Anal. Ed.*, 1942, **14**, 671.

[6] Krehl, W. A., Strong, F. M., and Elvehjem, C. A., *Ind. and Eng. Chem., Anal. Ed.*, 1943, **15**, 471.

[7] Krehl, W. A., Elvehjem, C. A., and Strong, F. M., *J. Biol. Chem.*, 1944, **156**, 13.

[8] Payne, D. S., *Chem. and Eng. News*, 1942, **20**, 1173.

[9] Zucker, T. F., and Zucker, L., *Ind. and Eng. Chem.*, 1943, **35**, 868.

TABLE I.
Summarized Analytical Data.

	Nicotinic acid						Pantothenic acid		
	HCl extraction			NaOH extraction					
Materials*	Samples used	Avg γ/g	Range γ/g	Samples used	Avg γ/g	Range γ/g	Samples used	Avg γ/g	Range γ/g
Sunflower seed meal	7	184	173-198	5	312	285-330	8	43	33-58
Wheat germ meal (A)	2	77	72-82	3	67	63-72	8	30	23-35
,, ,, ,, (B)	2	40	38-42	3	39	38-40			
Corn germ meal	5	38	35-40	5	35	34-36	8	25	24-26
Soybean meal	4	27	26-29	4	24	23-26	9	43	34-59
,, ,, (expeller)	2	17	16-19	2	17	17-18	10	26	19-32

* Furnished by Archer-Daniels-Midland Company, Minneapolis, Minn.; Central Soya Company, Decatur, Ind.; and VioBin Corporation, Monticello, Ill.

proximately 60% greater than that of soybean meal produced by an expeller process. The corn germ and wheat germ have less pantothenic acid than the sunflower seed meal. This is probably due to inherent differences between these materials and not to differences in the manufacturing processes because the corn germ, one lot of wheat germ, and the sunflower seed were subjected to the same conditions in the removal of the oil and in the subsequent treatment.

Summary. Sunflower seed meal is an excellent source of nicotinic acid. The content of pantothenic acid is equal or superior to that of soybean meal, wheat germ or corn germ.

15581

Chemical Derivatives of Gramicidin.

H. FRAENKEL-CONRAT, J. C. LEWIS, K. P. DIMICK, B. EDWARDS, H. C. REITZ, R. E. FERREL, B. A. BRANDON, AND H. S. OLCOTT.

From the Western Regional Research Laboratory, Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, U. S. Department of Agriculture, Albany, Cal.

Gramicidin has been found to react readily with formaldehyde to yield a product of improved pharmaceutical properties.[1] A study of the reaction mechanism indicated that formaldehyde was added in the form of hydroxymethyl groups quantitatively to the indole rings of the tryptophane,[2] which occurs to the extent of 40% in gramicidin. It was the object of the present investigation to search for other reagents which might, like formaldehyde, reduce the hemolytic and toxic but not the antibiotic activity of gramicidin, and at the same time render the derivative more water-soluble.

Reaction with Various Aldehydes. Representative results obtained with a series of aldehydes are summarized in Table I, Section A. Nitrogen analyses indicated that all aldehydes used combined with gramicidin, though to a variable extent. Acetal and glucose did not react appreciably. In contrast to the selective effect of formaldehyde, which decreases the hemolytic but not the antibacterial activity, all other aldehydes appeared to affect both activities to a similar degree. The aliphatic aldehydes generally caused a slight reduction, but glyoxal and

---

[1] Lewis, J. C., Dimick, K. P., Feustel, I. C., Fevold, H. L., Olcott, H. S., and Fraenkel-Conrat, H., *Science*, 1945, **102**, 274.

[2] Fraenkel-Conrat, H., Brandon, B. A., and Olcott, H. S., *Fed. Proc.*, 1946, **5**, 134; in press.

TABLE I.
Physical and Biological Properties of Gramicidin Derivatives.

Treatment	Nitrogen %	Equivalent of reagent introduced*	Solubility mg/100 ml†	Activity, % of original	
				Antibacterial	Hemolytic
		Section A.			
None	14.5	—	0.6	100	100
Formaldehyde	13.7	19	2.6	96	13
Acetaldehyde	13.7	13	1.3	64	88
Glyoxal (at 23°)	13.2	14	1.7	36	40
Glyoxylic acid	13.5	10	6.5 (>25)	79	82
Same, followed by formaldehyde	13.6	—	2.8	76	25
Tigaldehyde§	13.6	8	2.5	82	100
Benzaldehyde§	12.9	10	3.0	23	31
p-Dimethylamino benzaldehyde§	13.7	4	1.8	15	10
Acetal§	14.0	—	1.5	97	100
Glucose§	14.2	1	0.6	97	100
		Section B.			
Sulfuric acid conc.§	10.8	5		12	10
Pyridine-chlorosulfonic acid	9.9	18	(9.1)	41	14
		Section C.			
Acetic anhydride in acetic acid‡	13.7	10	0.1	104	100
Same, after formaldehyde	12.6	19.5	0.0	38	60
Acetic anhydride in pyridine	13.9	6	0.6	59	92
Same, after formaldehyde	12.2	24	0.5	22	15
Succinic anhydride in pyridine	13.6	7	1.1 (3.8)‖	85	40
Same, after formaldehyde	11.0	25	1.0 (>25)	24	4
Same, followed by formaldehyde	13.4	2.2-4.1	2.3 (5.6)	75	10

* Per 10^4 g of the original gramicidin, calculated from the nitrogen content for aldehydes, determined analytically for other derivatives.

† In 25% alcohol, 0.125 *M* sodium chloride; values in parenthesis represent solubility of the sodium salts.

‡ Reacted for 4 hours at 23°C.

§ These reactions were performed only once; all others at least twice. The data represent average values.

‖ (>25 in the absence of salt.)

the aromatic aldehydes caused marked losses. Determinations of the solubility in 25% alcohol indicated that all derivatives were more soluble than gramicidin, the glyoxylic acid-treated product exceeding in solubility the formaldehyde derivative, methylol-gramicidin. Furthermore, the glyoxylic acid derivative which contained up to 8 free acid groups per 10^4 g, yielded a neutral sodium salt which was not precipitated from concentrated alcoholic solution by the addition of much water. This product thus would appear to be of great potential usefulness except for the fact that its hemolytic and, presumably, its toxic activity was almost as high as that of unmodified gramicidin. Repeated attempts to reduce the hemolytic activity by treatment with formaldehyde before or after treatment with glyoxylic acid were not successful. It appeared that the glyoxylic acid residues were replaced by methylol groups, since the products had the desired low hemolytic activity, but only about one-fifth of the original acid groups. They were consequently less soluble in water at neutrality. The retention of hemolytic potency by the glyoxylic acid derivative was attributed to incomplete substitution of its indole groups. In view of the instability and commercial unavailability of glyoxylic acid, attempts were made to prepare more completely reacted glyoxylic acid derivatives by treating gramicidin with glyoxal, and subsequently oxidizing any free aldehyde groups with hydrogen peroxide, but without success.

Reaction with Sulfuric Acid and Pyridine-Chlorosulfonic Acid. Attempts were then made to obtain water-soluble derivatives of gramicidin by means of sulfation reactions (Table I, Section B). Concentrated sulfuric acid had been found to yield acid sulfate esters with hydroxyl groups of proteins,[3] and the addition product of chlorosulfonic acid with pyridine introduced sulfate groups into the indole groups as well.[4] Gramicidin had been used as a model substance in the elucidation of these reactions. When the derivative obtained with cold concentrated sulfuric acid was assayed, it was found to be low both in hemolytic and antibacterial activity; sulfation of methylol-gramicidin by this technic gave, against expectation, a highly insoluble product.

Pyridine-chlorosulfonic acid, on the other hand, yielded derivatives, which, isolated as the barium or sodium salts, were usually soluble in dilute aqueous alcohol in the absence of salt, but not in water. These products retained varying fractions of the original antibacterial activity, and from 10 to 38% of the hemolytic activity. Thus it appears that the introduction of sulfate radicals into the indole groups of gramicidin, while being somewhat harder to control, affects the 2 biological activities in the same direction as does the introduction of methylol groups. On the other hand sulfation may cause more marked increases in solubility of gramicidin. Sulfation of methylol-gramicidin by the same technic gave a product of low activity and solubility in all solvents.

Reaction with Acid Anhydrides (Table I, Section C). Treatment of gramicidin with acetic anhydride in *glacial acetic acid* at temperatures up to 40° and for reaction periods up to 3 days led to the introduction of increasing amounts of acetyl. The highest value (18 equivalents per 10^4 g material, after correction for the blank values of 5.0 equivalents of volatile acid liberated from gramicidin) was of the same order as the total number of indole groups (19.4) and considerably in excess of the hydroxyl groups (4.5) present in gramicidin. Thus it appears that at least part of the indole groups are acetylated under these conditions. Similar treatment of methylol-gramicidin led to the introduction of up to 23 equivalents of acetyl. Assays of the acetylated derivatives obtained at room temperature showed no loss in activity for that derived directly from gramicidin; and some decrease in antibacterial and regeneration of hemolytic activity for the derivative of methylol-gramicidin. In contrast to gramicidin, most simple indoles cannot readily be N-acetylated; 5-methoxyindole is an exception.[5] However, Toennies and Kolb have indicated that acetic anhydride in the presence of perchloric acid reacts readily with tryptophane to form the indole-N-acetyl derivative.[6]

An alternate technic of acetylation was suggested by Hotchkiss' observation[7] that acetic anhydride in pyridine reacts exclusively with the hydroxyl groups of gramicidin, thus permitting the use of Stodola's acetylation technic[8] as a measure of its hydroxyl groups. This method was also found applicable in determining the number of methylol groups introduced into gramicidin through formaldehyde treatment.[2] Assays of gramicidin and methylol-gramicidin, O-acetylated by this technic, showed the former to have lost part of its antibiotic, but none of its hemolytic activity, while the latter was greatly lowered in both respects. None of the acetyl derivatives were more water-soluble than the starting material.

The readiness with which this reaction occurred suggested that it might lead to a water-soluble product if succinic anhydride were used instead of acetic anhydride. The extent of combination of succinic anhydride with gramicidin could readily be ascertained by titration of the acid groups introduced into the nonpolar polypeptide. In good agreement with the acetyl values, 10^4 g of gramicidin bound 7 equivalents of succinic anhydride, and 10^4 g of methylol-gramicidin bound about 25 equivalents of succinic anhydride. The succinyl derivative of gramicidin retained about 80% of the original antibacterial and 40% of the hemolytic activity; as the sodium salt it gave a colloidal 2% solution in distilled water and a clear solution in 5% ethyl alcohol. However, in the presence of other electrolytes its solubility was only slightly higher than that of the methylol-gramicidin; as the free acid, it had low solubility in water. The hemolytic activity of this product could be lowered by subsequent formaldehyde treatment. This, however, led to partial hydrolysis of the succinic ester groups, even if the reaction was performed under gentle conditions (Table I, Section C).

---

[3] Reitz, H. C., Ferrel, R. E., Fraenkel-Conrat, H., and Olcott, H. S., *J. Am. Chem. Soc.*, 1946, **68**, 1024.

[4] Reitz, H. C., Ferrel, R. E., Olcott, H. S., and Fraenkel-Conrat, H., *J. Am. Chem. Soc.*, 1946, **68**, 1031.

[5] Blaikie, K. G., and Perkin, W. H., Jr., *J. Chem. Soc.*, 1924, 296.

[6] Toennies, G., and Kolb, T. T., *J. Biol. Chem.*, 1943, **144**, 219.

[7] Hotchkiss, H. D., *J. Biol. Chem.*, 1941, **141**, 171.

[8] Stodola, F., *Mikrochemie*, 1937, **21**, 180.

The succinyl derivative of methylol-gramicidin had 24% of the original antibacterial, and only 1 to 5% of the hemolytic activity. Its sodium salt was freely soluble in water (5% solutions were prepared), even in the presence of sodium chloride. However, hydrolysis of ester bonds occurred at an appreciable rate, and this led to a gelling of concentrated aqueous solutions within a few hours at room temperature.

The observed increases in water solubility and decreases in hemolytic activity achieved by reaction of gramicidin or methylol-gramicidin with succinic anhydride were accompanied by marked decreases (95 to 98%) in the toxicity of the products toward rats. This was particularly surprising in the case of succinic acid-treated gramicidin, which was not toxic when given in dosages ranging up to 40 times the lethal dose of the original gramicidin, notwithstanding the fact that the derivative retained, as stated above, about 40% of the original *in vitro* hemolytic activity. The derivatives obtained by consecutive treatments with formaldehyde and succinic anhydrides and the toxicity data have been the subject of a preliminary note.[9]

Methods and Materials. Gramicidin was kindly supplied by the Wallerstein Laboratories.* All reagents were commercial preparations, with the exception of glyoxylic acid, which was prepared according to Doebner.[10]

The conditions used for treating gramicidin with the various aldehydes were those found favorable in the case of formaldehyde.[1] Sodium carbonate, instead of sodium hydroxide, was used in part of the experiments as an alkaline buffer. When 100 mg of gramicidin, dissolved in 2.5 ml of 95% ethanol containing 200 mg of sodium carbonate, was treated for 2 days at 53° with 1 ml of 40% aqueous formaldehyde, the isolated product corresponded in all respects to that obtained in the usual manner. Acetaldehyde treatment in the presence of sodium carbonate led to a brown impure product, while a white product was obtained in the presence of sodium hydroxide.

The technics of sulfation with concentrated sulfuric acid and pyridine-chlorosulfonic acid have previously been described.[3,4] Acetylation with acetic anhydride was performed either in pyridine according to Stodola (one hour at 95°),[8] or in 50% solution in glacial acetic acid at temperatures of 20 to 40° for time periods up to 3 days. Succinic anhydride was used successfully only in pyridine, because of its limited solubility in glacial acetic acid. Similar products were obtained when the reactions were performed for 18 hours at 40 or 53°, or for one hour at 78 or 95°.

---

[9] Olcott, H. S., Lewis, J. C., Dimick, K. P., Fevold, H. L., and Fraenkel-Conrat, H., *Arch. Biochem.*, 1946, **10**, 553.

* Part of the gramicidin used in these studies was prepared in this laboratory by a method which depends upon the selective precipitation of tyrocidin and tyrocidin-like peptides from tyrothricin solutions by means of phosphotungstic acid. For example, to 50 g of tyrothricin in 650 ml of absolute alcohol was added 85 ml of a 60% solution of phosphotungstic acid in the same solvent. The precipitate was centrifuged off and washed once with 100 ml of absolute alcohol. The combined soluble fractions were precipitated by the addition of 4 liters of water and sodium chloride, if necessary, to coagulate the suspension. The crude gramicidin precipitate was collected by centrifugation, suspended in about 200 ml of water, brought to pH 10 by the addition of 1 N sodium hydroxide, and allowed to stand overnight (to decompose the phosphotungstate). The gramicidin was centrifuged off and washed repeatedly by resuspension and recentrifugation to remove traces of tungsten. Salt or ammonium carbonate was added to coagulate milky suspensions when these could not be separated by centrifugation. The final dried product weighed 10.5 g (21% of the tyrothricin) and appeared to contain approximately 5% tyrocidin by an *in vitro* hemolytic assay. A sample of pure gramicidin treated by this procedure was found to have unchanged chemical and biological properties.

[10] Doebner, O., *Ann. der Chem.*, 1900, **311**, 129.

All derivatives, except those sulfated or acetylated on the indole nitrogen, were isolated by precipitation from acid solution with excess water. The products were washed repeatedly with water, redissolved in alcohol and again precipitated with water. Acetic acid served for acidification and in many instances also for flocculation of the products; in other cases, sodium chloride was used in amounts up to 0.1 M. Isolation was easily accomplished because of the fact that gramicidin and all of its derivatives are only very slightly soluble in acid aqueous media. The water-soluble derivatives obtained in the course of this study were all sodium salts, soluble only in neutral aqueous solution. The product obtained with pyridine-chlorosulfonic acid had to be isolated from neutral solution as the sodium or barium salt, because the indole-sulfate bond was unstable in acid solution.[4] This was achieved by removing excess sulfate as barium sulfate, pyridine by evaporation from alkaline solution, and neutral inorganic matter by extraction with water. The gramicidin derivative was subsequently extracted with 50% aqueous alcohol. Nitrogen and tryptophane analyses supplied a reliable measure of the purity of the product. The indole-acetylated derivative also was unstable upon dilution of the acetic acid reaction mixture with water. It was isolated by evaporation of all volatile matter under a high vacuum.

Tryptophane analyses were performed by the Horn and Jones method[11] after hydrolysis; 5 to 10 mg were heated for 20 hours at 100° with 0.8 ml of glacial acetic acid and 3.0 ml of 6 N hydrochloric acid in evacuated Thunberg tubes, which had been flushed with CO_2. The tryptophane content of pure gramicidin was found to be 38.1%.[†] Benzoyl-d,l-tryptophane after similar hydrolysis contained 63.9% tryptophane; calculated 66.1%. Table II shows the apparent tryptophane content of the various derivatives of gramicidin.

Analyses of intact gramicidin in 50% acetic acid solution by the Horn and Jones method indicated about 40% tryptophane, but the value varied from 35 to 45%, depending upon the amount of gramicidin used, and thus had no absolute quantitative significance.

The spectrophotometric analyses occasionally performed did not necessitate hydrolysis; they indicated a tryptophane content of 39.6% for gramicidin.

Hydroxyl groups were determined by quantitative acetylation[8] (Table II). Bound acetyl groups were determined after hydrolysis for 3 days at 100° in evacuated Thunberg tubes with 6 N sulfuric acid, followed by steam distillation and titration of the distillate.[12] Sulfate groups were determined after hydrolysis as barium sulfate.[3] The acid groups introduced through glyoxylic acid or succinic anhydride treatments were titrated in alcoholic solutions with aqueous 0.1 N alkali with phenolphthalein as indicator. The Kjeldahl method was found to give the expected tryptophane nitrogen values when mercuric oxide was used as catalyst.[14]

Assays for hemolytic and antibacterial activity were performed by the methods described in the previous publication.[1]

To determine the solubility of the products, samples of 2.5 mg were dissolved in 2.5 ml of absolute alcohol, then treated with 8.25 ml of water and 1.25 ml of 1 M sodium chloride. The precipitates were centrifuged off after 15 to 30 minutes. The amount of gramicidin remaining in solution was de-

---

[11] Horn, M. J., and Jones, D. B., *J. Biol. Chem.*, 1944, **157**, 153.

[†] Hotchkiss[7] found 37.3%. Synge[13] gave 38.6 and 41.1% as the corrected (by 10%) values for two recrystallized preparations.

[12] Blackburn, S., and Phillips, H., *Biochem. J.*, 1944, **38**, 171.

[13] Synge, R. L. M., *Biochem. J.*, 1944, **38**, 285.

[14] White, L. M., and Secor, G. E., *Ind. Eng. Chem., Anal. Ed.*, 1946, **18**, 457.

TABLE II.
Chemical Properties of Gramicidin Derivatives.

Reagent	Equivalents introduced*		Equivalents of tryptophane found colorimetrically*	
	Reagent	Hydroxyl	With hydrol.	Without hydrol.†
Untreated	—	—	19.4	20
Formaldehyde	19	19	about 5‡§	<5§
Acetaldehyde	13	0	17.2	18
Tigaldehyde	8	2	15.2	
Benzaldehyde	10	3	15.4	18
Glyoxal	14	4	9.7	
Sulfuric acid	5	—	16.9	
Pyridine-chlorosulfonic acid	18	—	16.3	
Acetic anhydride in acetic acid	18	—		20
Acetic anhydride in pyridine	6	—		20
Succinic anhydride in pyridine	7			18
Formaldehyde, then acetic anhydride (in pyridine)	24.5	—		<5§
Formaldehyde, then succinic anhydride	25	none		<5§
Acetic anhydride (in acetic acid), then formaldehyde		16.5		<5§
Succinic anhydride, then formaldehyde		24.8		<5§

* Per 10^4 g of the original gramicidin.
† Approximate values only are obtained without hydrolysis.
‡ Methylol gramicidin, upon hydrolysis, yields acid-insoluble humin.
§ Based on readings of an atypical purple color.

termined by means of spectrophotometric tryptophane analyses. This method alone is sufficiently sensitive to be applicable to the low concentrations; it has the further advantage over the colorimetric method that it appears to give true tryptophane values for substituted indole derivatives, such as methylol-gramicidin, which retains only about 25% of its original colorimetric value.[2] The introduction of benzene rings by means of benzaldehyde, however, led to high apparent tryptophane values for this derivative (127% of that calculated on the basis of nitrogen).

Discussion. The present investigation reveals the remarkable fact that a highly active natural product may be extensively substituted with a variety of chemical agents without undergoing great changes in some or all of its biological activities. Thus, the antibacterial activity of gramicidin is largely retained after substitution of all of its hydroxyl groups, and of all of its indole groups, either on the nitrogen or the carbon atoms, with alcoholic, acidic, or nonpolar side chains. This is even more astonishing, in view of the fact that the hydroxyl and indole groups appear to be the only reactive or polar centers of the gramicidin molecule. The extent of substitution achieved with the various classes of reagents approaches, but never exceeds, the number of equivalents expected to react. The foreign groups introduced may amount to as much as 20% of the weight of the gramicidin for some of the promising derivatives.

The above results might have been of greater general significance if systematic studies of the most favorable reagents and conditions, and complete bioassays, including therapeutic and toxicity studies, had not been outside of the scope of this brief survey of possibilities. The preliminary findings with succinyl derivatives showed that their *in vitro* hemolytic activities were not necessarily indicative of their *in vivo* toxicity. Since only one type of antibacterial and the hemolytic test, both *in vitro,* were used as primary criteria for successful modification, the possibility definitely exists that potentially useful products have remained unrecognized and were bypassed in the course of this study.

The mode of combination of the various reagents with gramicidin has in part been elucidated and demonstrated in previous studies.[2–4] All aldehydes might be assumed to combine with the indole groups, as does formaldehyde—that is, by addition to the nitrogen atom. However, far fewer hydroxyl groups seemed to be formed, or could be detected by acetylation, than corresponded to the amounts of aldehyde combined. The apparent tryptophane content, as determined colorimetrically, showed decreases of only 50% after glyoxal treatment, with values more closely approaching the original for other aldehydes. In contrast, combination with formaldehyde led to formation of an equivalent number of hydroxyl groups, and to a decrease of the apparent tryptophane content by 75%. The derivative obtained with pyridine chlorosulfonic acid had the expected full chromogenic value. This finding confirmed the conclusion that the sulfate had combined with the nitrogen, rather than with the 2-carbon atom.[2,4] The same was the case for the acetyl groups introduced into the indoles by means of acetic anhydride in acetic acid. The chromogenic value of the derivatives obtained with concentrated sulfuric acid or with acyl anhydrides in pyridine, reagents which combine only with the hydroxyl groups,[3,7] was similarly unchanged, when calculated on the basis of their nitrogen contents.

Summary. Gramicidin was treated with a number of reagents which were known, or could be shown to combine with, either its indole, or its hydroxyl groups, or both. The products were prepared and assayed in search of a water-soluble derivative of low hemolytic and high antibacterial activity. Formaldehyde had previously been shown to decrease selectively the hemolytic activity, but to render gramicidin only slightly less water-insoluble.

1. A glyoxylic acid derivative was obtained, the sodium salt of which was water-soluble. The product, however, retained most of the original hemolytic activity, and attempts to find experimental conditions under which the advantages of the glyoxylic and formaldehyde treatment could be combined were not successful.

2. An indole sulfamate of gramicidin was obtained with pyridine-chlorosulfonic acid, the salts of which were readily soluble in aqueous alcohol. The product showed somewhat greater decreases in hemolytic, than in antibacterial activity.

3. The succinic half esters of gramicidin and methylol gramicidin showed the greatest promise. The succinyl derivative of gramicidin, containing one succinyl group for each ethanolamine residue, gave a water-soluble sodium salt. Its hemolytic activity was slightly decreased and could be further lowered by subsequent formaldehyde treatment. Notwithstanding the *in vitro* hemolytic activity, the derivative appeared to be remarkably nontoxic by intravenous injection. The succinic anhydride derivative of methylolgramicidin, containing one succinyl for each indole-methylol plus each original hydroxyl group, was lowered in all, but markedly more in hemolytic and toxic than in antibacterial activities. Its sodium salt was water-soluble even in the presence of other ions, but not very stable at neutrality.

The authors are indebted to L. M. White and G. E. Secor for the elementary analyses. Antibacterial assays were kindly performed by B. M. Humphreys, and the preliminary toxicity assays by A. M. Ambrose.

15582 P

Effect of Thyroprotein upon Vitamin Content of Milk.

A. R. Kemmerer, R. A. Bolomey, M. G. Vavich, and R. N. Davis.

From the Departments of Nutrition and Dairy Husbandry, University of Arizona, Tucson, Ariz.

Graham[1] reported that the feeding of thyroxine to dairy cows in the declining phases of lactation caused a distinct rise in the amount of milk produced and in the percentage of butter fat. Synthetic thyroprotein (iodinated casein or protamone) has

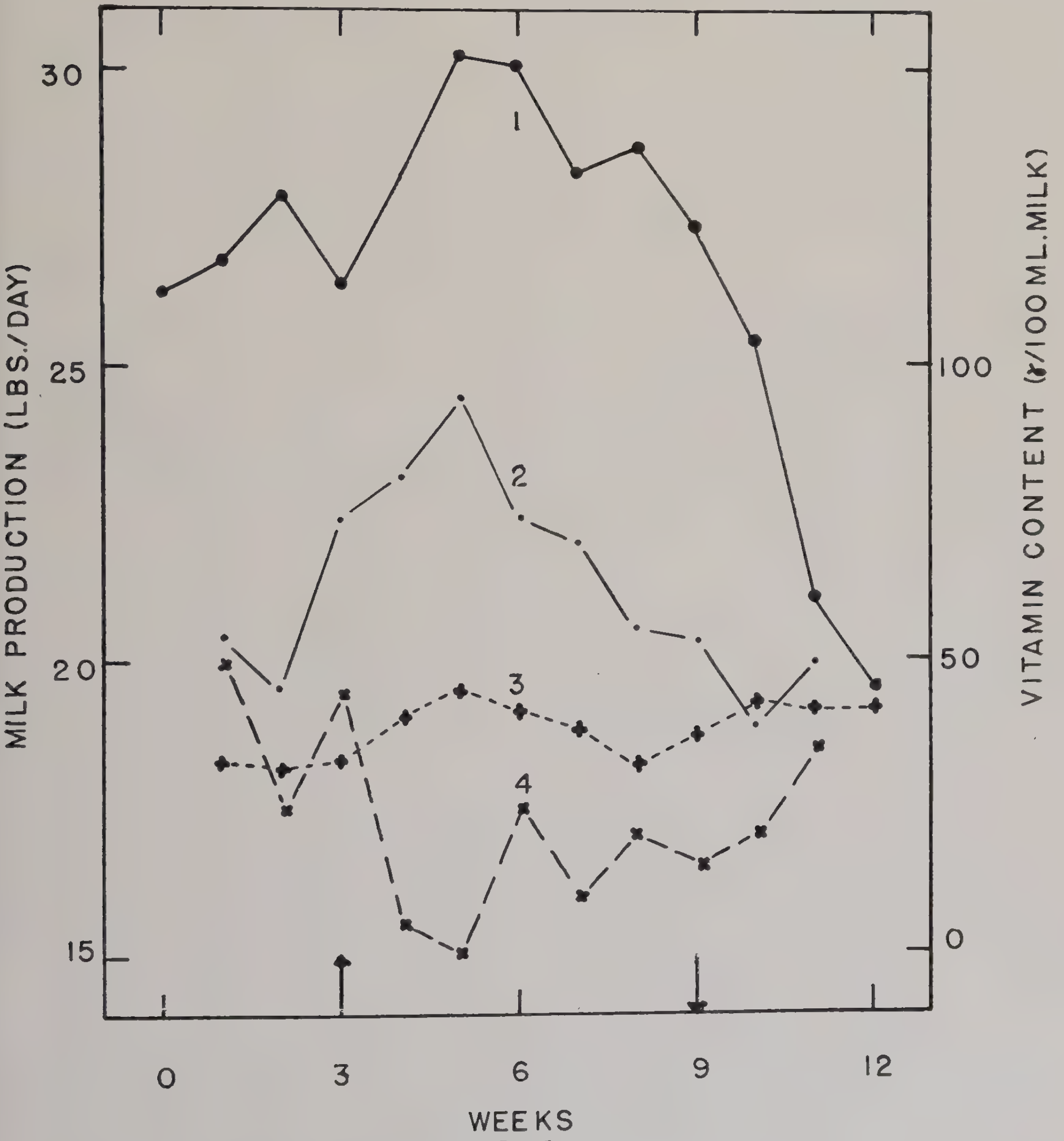


Fig. 1.

Effect of thyroprotein on milk production and on vitamin content of milk. (1) Milk production. (2) Niacin content. (3) Thiamin content. (4) Riboflavin content.

---

[1] Graham, W. R., Jr., *J. Nutr.*, 1934, **7**, 407.

been reported in a number of papers[2–6] to produce the same effect. Van Landingham *et al.*[6] found that feeding thyroprotein reduced the ascorbic acid content of milk approximately one-third. The present report deals with the effect of feeding thyroprotein upon the content of vitamin A, thiamin, riboflavin and niacin in milk.

Two pure bred Guernsey cows and 2 pure bred Holstein cows were fed a grain mixture supplemented with alfalfa hay. The amount of feed was kept constant for each cow during the entire experimental period of 12 weeks. At the start of the experiment the cows were fed the regular ration, for 3 weeks, then for 6 weeks the regular ration plus 15 g iodinated casein* daily, and for the remaining 3 weeks the regular ration again.

Thiamin was determined by the method of Hodson,[7] niacin by the method of Krehl, Strong and Elvehjem,[8] and riboflavin by the method of Hand[9] modified according to McLaren, Cover and Pearson.[10] Vitamin A and carotene were determined by the method of Berl and Peterson.[11]

The average results obtained with the 2 Guernsey cows are given graphically in Fig 1. As in previous reports[2–6] the feeding of thyroprotein caused a rise in milk production which lessened during the latter part of the period of thyroprotein feeding. In addition the concentration (γ/ml) of niacin and thiamin in the milk was increased while that of riboflavin was decreased. It is interesting that the maximum concentration of niacin and thiamin and the minimum concentration of riboflavin were reached at the peak of the milk production. The rise in thiamin content was comparatively very small and not significant. On the 12th week it was only slightly less than the peak value. The rise in niacin was comparatively large and highly significant. Curves showing the effect of the thyroprotein on the concentration of vitamin A and carotene in the milk are not included in Fig. 1 since no significant changes were observed. The results obtained with the Holstein cows closely approximated those with the Guernseys.

---

[2] Reineke, E. P., *J. Dairy Sci.*, 1942, **25**, 201.

[3] Reineke, E. P., *J. Dairy Sci.*, 1943, **26**, 750.

[4] Reineke, E. P., and Turner, C. W., *J. Dairy Sci.*, 1942, **25**, 393.

[5] Reese, R. P., *J. Dairy Sci.*, 1944, **27**, 545.

[6] Van Landingham, A. H., Henderson, H. O., and Weakley, C. E., Jr., *J. Dairy Sci.*, 1944, **27**, 385.

* Acknowledgment is made to W. R. Graham, Jr., Cerophyll Laboratories, Inc., for the generous supplies of iodinated casein.

[7] Hodson, A. Z., *Food Research*, 1945, **10**, 351.

[8] Krehl, W. A., Strong, F. M., and Elvehjem, C. A., *Ind. Eng. Chem., Anal. Ed.*, 1943, **15**, 471.

[9] Hand, D. B., *Ind. Eng. Chem., Anal. Ed.*, 1939, **11**, 306.

[10] McLaren, B., Cover, S., and Pearson, P. B., *Arch. Biochem.*, 1944, **4**. 1.

[11] Berl, S., and Peterson, W. H., *J. Nutr.*, 1943, **26**, 527.

15583

Plasma Tocopherol Levels in Sprue.*

William J. Darby, Mary Ellen Cherrington, and Julian M. Ruffin.
(Introduced by C. S. Robinson.)
From the Departments of Biochemistry and Medicine, Vanderbilt University School of Medicine, Nashville, and the Department of Medicine, Duke University School of Medicine, Durham.

The steatorrhea of sprue is accompanied by low levels of vitamin A and carotene in the blood plasma,[1,2] by hypoprothrombinemia

---

* This work was supported by grants from Distillation Products, Inc., the Nutrition Foundation, the International Health Division of The Rockefeller Foundation, and by the Tennessee State Department of Public Health. The latter 3 grants were through the Tennessee-Vanderbilt Nutrition Project.

[1] Ingelfinger, F. J., *New Eng. J. Med.*, 1943, **228**, 180.

[2] Cayer, D., Ruffin, J. M., and Perlzweig, W. A., *Am. J. Med. Sci.*, 1945, **210**, 200.

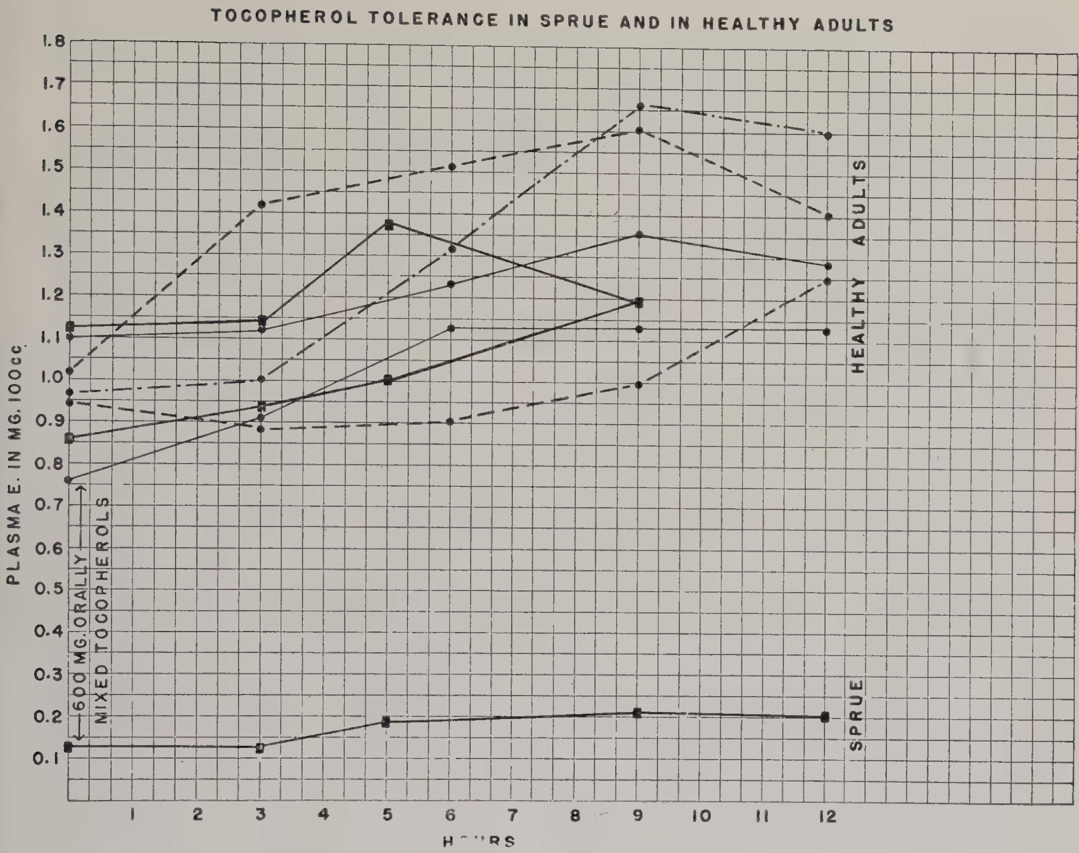


Fig. 1.

of vitamin K deficiency,[3,4,1] by a flattening of the glucose tolerance curve, and by deranged calcium metabolism[5] perhaps in part due to vitamin D deficiency. Studies on rats with bile fistulas indicate that the absorption of fat-soluble vitamin E is dependent upon the presence of normal fat absorption.[6] The plasma tocopherol level in sprue does not appear to have been investigated, but Minot[7] found a relatively low value in a child with celiac disease.

We have recently studied the plasma tocopherol levels[8,9] in 20 patients with sprue in relapse or remission. The lowest values have occurred in those patients who were in relapse or early remission. In one of these patients (J.D.) the tocopherol level gradually decreased to a very low value as the patient underwent a relapse following the withholding of pteroylglutamic acid (PGA) therapy, and is again increasing after reinstitution of therapy with PGA. During relapse a maximum rise in serum concentration of tocopherols of 0.09 mg per 100 cc was noted after an oral dose of 600 mg of mixed tocopherols, in contrast to the mean maximal rise of 0.37 mg in 7 healthy adults after a similar dose. These changes are depicted

---

[3] Fanconi, *Deut. Med. Wochensch.*, 1938, **64**, 1565.

[4] Butt, H. R., and Snell, A. M., *Vitamin K*, Philadelphia, 1941, 101.

[5] Bennett, T. I., Hunter, D., and Vaughan, J. M., *Quart. J. Med.*, 1932, **1**, 603.

[6] Greaves, J. D., and Schmidt, C. L. A., PROC. SOC. EXP. BIOL. AND MED., 1937, **37**, 40.

[7] Minot, A. S., *J. Lab. Clin. Med.*, 1944, **29**, 772.

[8] Quaife, M. L., and Harris, P. L., *J. Biol. Chem.*, 1944, **156**, 499.

[9] Quaife, M. L., and Biehler, R., *J. Biol. Chem.*, 1945, **159**, 663.

TABLE I.
Plasma Tocopherol Levels in Sprue in Relapse and Early Remission Following Therapy with Pteroylglutamic Acid (PGA).

Case	Date	Plasma tocopherol, mg/100 cc	Remarks
J.D.	5-1-46	0.76	Clinical remission after PGA therapy.
	6-19-46	0.30	Clinical and hematologic relapse.
	7-13-46	0.14	,, ,, ,, ,,
	7-16-46	0.12	,, ,, ,, ,,
	7-25-46	—	Therapy with PGA reinstituted.
	9-4-46	0.34	Clinical remission after PGA therapy.
W.O.	4-27-46	0.25	In relapse.
	5-6-46	—	Therapy instituted with PGA.
	5-16-46	0.43	Remission.
	7-13-46	0.43	,,
P.B.	6-5-46	0.32	Clinical remission following therapy with PGA from November, 1945.
	7-16-46	0.36	

in Fig. 1. In another patient (W.O.) there was a slight increase in plasma tocopherol concentration following the administration of pteroylglutamic acid. The concentration of serum carotene has tended to parallel the tocopherol levels in these cases. Low values of serum vitamin E have also been found in an infant with cystic fibrosis of the pancreas and in some patients having liver disease, either hepatitis or cirrhosis.

The mean value of plasma tocopherol in 10 apparently healthy adults was 1.06 ± 0.06 mg per 100 cc. These results are in agreement with those of other workers.[7,8] In 140 determinations on patients having either pernicious anemia or acromegaly, and in pregnancy, we have not encountered any value of plasma tocopherol below 0.62 mg per 100 cc.

In 17 patients with sprue in remission following a long period of treatment with liver extract, the tocopherol levels were found to be essentially normal, the mean value being 0.97 ± 0.08 mg per 100 cc.

The possible significance of these low values during a relapse of sprue is underscored by the reports[10,11] that muscle tissue at autopsy from patients with sprue showed pigment resembling that seen in vitamin E deficient animals. Furthermore, experimental studies reveal that sulfa-treated rats become deficient in vitamin M and sometimes manifest tocopherol deficiency.[12] It seems probable that a deficiency of some members of the vitamin M complex may affect the absorption or utilization of fat-soluble vitamins. In seeking evidences of tocopherol deficiency in man it may be fruitful to study carefully active cases of steatorrhea.

Summary. Three patients with sprue in relapse or early remission were found to exhibit very low plasma tocopherol levels. The rise in plasma concentration of tocopherols following an oral dose of 600 mg was less in a patient with sprue in relapse than in 7 healthy subjects. The average concentration of plasma tocopherols was essentially normal in 17 sprue patients treated with liver extract for long periods. These findings are of interest in view of the recognized occurrence of deficiencies of other fat-soluble vitamins in sprue.

---

[10] Pappenheimer, A. M., and Victor, J., *Am. J. Path.*, 1946, **22**, 395.

[11] Adlersberg, D., and Schein, J. T., reported at the San Francisco Meeting of the American Medical Assn., Section on Gastroenterology, July 3, 1946.

[12] Daft, F. S., Endicott, K. M., Ashburn, L. L., and Sebrell, W. H., PROC. SOC. EXP. BIOL. AND MED., 1943, **53**, 130.

15584

Studies on Malarial Parasites. V. Effects of Ascorbic Acid on Malaria (*Plasmodium knowlesi*) in Monkeys.*

RALPH W. MCKEE AND QUENTIN M. GEIMAN. (Introduced by A. B. Hastings.)

From the Departments of Biological Chemistry and of Comparative Pathology and Tropical Medicine, Harvard Schools of Medicine and Public Health, Boston, Mass.

Several different investigators[1-3] have reported that in human cases of tertian malaria there was an increased demand for ascorbic acid and the administration of additional vitamin was necessary to prevent a mild state of scurvy. In spite of these observations no one has reported a causal relationship between malaria and ascorbic acid deficiency.

During the course of our malaria studies with *P. knowlesi* in monkeys *(Macaca mulatta)*[4] certain observations were made which led us to suspect that ascorbic acid might have some influence on the course of parasitemia in the animal host. Using the method of Mindlin and Butler[5] for determining plasma ascorbic acid and of Butler *et al.*[6] for whole blood levels of the vitamin it was found that the average plasma level of ascorbic acid for 20 parasitized monkeys was only 0.24 mg %. Nonparasitized animals were found to have an average plasma level of 0.56 mg %. Also during the course of our studies on 120 monkeys, 7 animals inoculated with parasites showed no plasma ascorbic acid and less than 0.1 mg % in the whole blood. This condition was present in spite of the fact that the animals were receiving a well balanced diet containing generous daily portions of raw potatoes and oranges. Such a condition has been previously reported in both humans[7] and monkeys.[8] This may be explained on the basis of an abnormal intestinal flora since it has been shown by various workers[9,10] that under certain conditions some intestinal bacteria destroy ascorbic acid and may be responsible for a state of scurvy.

The 7 animals having extremely low blood ascorbic acid values showed abnormal courses of parasitemia. Instead of the usual rapid increases in numbers of parasites and death of the animals within 6 or 7 days after inoculation there was only a slow rise in percentage of parasites, and a gradual spontaneous control of the infection. Into 4 of these animals ascorbic acid was injected intramuscularly on 3 successive days (starting 7, 7, 8 and 9 days following parasite inoculation in the 4 monkeys) and in all cases a significant increase in numbers of circulating parasites was observed. One of these animals (given ascorbic acid 7 days after parasite inoculation) then showed a normal rise in parasite count and died in the usual manner with a high percentage of the erythrocytes parasitized. The other 3 animals (given ascorbic acid 7, 8 and 9 days after parasite inoculation) however, showed a subsequent drop in parasite count with spontaneous control of the infection, presumably due to the development of an immunity prior to the injection of ascorbic acid. The remaining 3 monkeys out of the total 7 were given no vitamin treatment and all 3 completely controlled their infections. In Ta-

---

* This work was done under a contract recommended by the Committee of Medical Research, between the Office of Scientific Research and Development and the President and Fellows of Harvard College.

[1] Mohr, W., *Abstr. Trop. Dis. Bull.*, 1943, **40**, 13.

[2] Lotze, H., *Abstr. Trop. Dis. Bull.*, 1938, **35**, 733.

[3] Krishman, K. V., *Trop. Dis. Bull.*, 1940, **37**, 744.

[4] Ball, E. G., Anfinsen, C. B., Geiman, Q. M., McKee, R. W., and Ormsbee, R. A., *Science*, 1945, **101**, 542.

[5] Mindlin, R. L., and Butler, A. M., *J. Biol. Chem.*, 1938, **122**, 673.

[6] Butler, A. M., Cushman, M., and MacLachlan, E. A., *J. Biol. Chem.*, 1943, **150**, 453.

[7] Marin, P., *Minerva Med.*, 1936, **2**, 25.

[8] Horvitt, B. F., *Arch. Path.*, 1931, **11**, 574.

[9] Young, R. M., and Rettger, L. F., *J. Bact.*, 1943, **46**, 351.

[10] Kendall, A. I., and Chinn, H., *J. Inf. Dis.*, 1938, **62**, 330.

TABLE I.
Effect of Ascorbic Acid on Parasitemia of Ascorbic Acid Deficient Monkeys (Spontaneously Appearing).

Days after parasite inoculation	Total parasite count		
	Ascorbic acid deficient animals		
	No. 59	No. 101	No. 109
4	—	0.1	—
5	0.1	—	—
6	0.3	2.4	occasional parasite in thick film
7	0.5	0.8*	—
8	0.5	8.5*	0.004
9	—	20.6*	0.004*
10	2.0	13.5	1.1*
11	2.4	dead	0.05*
12	0.4		—
13	occasional parasite in thick film (chronic)		0.01 (chronic)

* 80-100 mg Merck's crystalline ascorbic acid intramuscularly. All 10 animals referred to were approximately the same size with an average weight of about 2.5 kilos.

ble I are shown the parasite changes of 3 representative animals of the 7.

Three monkeys were then placed on a synthetic diet deficient in ascorbic acid similar to that of Shaw and coworkers.[11] This diet which was freshly mixed every 5 or 6 days and stored in the cold room at +2°C consisted of the following percentages of ingredients: sucrose 65, casein 15, Phillips and Hart IV salts 4, corn oil 4, cod liver oil 2, and yeast 10.[†] Vitamins were supplemented in the indicated numbers of milligrams per kilogram of diet: biotin 0.1, thiamine 5, riboflavin 5, pyridoxine 5, calcium pantothenate 15, nicotinic acid 25, choline 250, inositol 500, and paraaminobenzoic acid 500. When early deficiency symptoms appeared (disappearance of plasma and whole blood ascorbic acid, slight loss of body weight and increased capillary fragility), the animals were inoculated intravenously with 6 million parasites per kg of body weight. A control animal on a normal diet containing adequate ascorbic acid was likewise inoculated with the same dosage of parasites. To the 3 experimental animals, No. 120, 119 and 117, a solution of crystalline ascorbic acid was injected, as shown in Table II, starting, respectively, immediately before, 4 days after and 14 days after parasite inoculation. The control and the animal No. 120 showed immediate parasite increases with normal courses of parasitemia and deaths on the 7th day with, in each case, 31% of the erythrocytes parasitized. Animal No. 119 showed no parasite increase until after ascorbic acid was given but then had a normal course of parasitemia, with death on the 6th day following vitamin administration. Animal No. 117 showed only a small parasite increase prior to ascorbic acid administration, but died 2 days after receiving vitamin with only 8% of the erythrocytes parasitized. This animal did not die of the malaria infection alone, but manifested a complicating anemia perhaps due to the more advanced state of the vitamin deficiency and a greatly lowered food intake during the last week of the animal's life. (There appeared also to be some resistance to parasite increase indicative of an immune reaction).

Whether this action of ascorbic acid administration to the vitamin-deficient animals is a direct effect on the parasites or if it is an indirect one through the animal host we do not know. We suspect that it may be the latter since attempts to demonstrate the need of ascorbic acid for the *in vitro* growth and multiplication of *P. knowlesi* in

---

[11] Shaw, J. H., Phillips, P. H., and Elvehjem, C. A., *J. Nutrition*, 1945, **29**, 365.

[†] Generous quantities of Strain K Dried Brewer's Yeast were kindly supplied by Anhauser-Busch, Inc., St. Louis, Mo.

TABLE II.
Effect of Ascorbic Acid on Parasitemia of Ascorbic Acid Deficient Monkeys (Produced by Dietary Deficiency).

Days after parasite inoculation	Total parasite count			
	Control animal (normal diet)	Ascorbic acid deficient animals (Vitamin C deficient diet)		
		No. 120	No. 119	No. 117
0	—	—‡	—	—
3	0.3%	0.3%‡	350/mm^3	50/mm^3
4	2.0 ”	2.7 ”‡	270/ ”‡	120/ ”
5	10.0 ”	8.0 ”‡	0.2 %‡	30/ ”
6	12.6 ”	14.9 ”‡	0.15 ”‡	150/ ”
7	31.3 ”*	31.0 ”*	0.8 ”‡	100/ ”
8			3.2 ”‡	300/ ”
9			26.0 ”	580/ ”
10			144.7 ”*†	2000/ ”
11				0.5%
12				1.2 ”
13				0.9 ”
14				5.5 ”‡
15				8.8 ”*‡

† 84% of the red cells contained parasites with many double and triple infections per cell.
* Animal died within 18 hours following this parasite count.
‡ 80-100 mg Merck's crystalline ascorbic acid intramuscularly.

24-hour cultures have so far been inconclusive, although in the absence of the vitamin the usual protoplasmic mass is not achieved. This growth deficiency and the degenerate appearance of the parasites are also very striking in the vitamin-deficient host. These studies on the effect of ascorbic acid and of ascorbic acid analogs are being continued with simian and human plasmodia.

Conclusions and Summary. 1. Plasma and whole blood ascorbic acid levels were significantly lower in parasitized monkeys than in normal animals.

2. There was an abnormal course of parasitemia in 7 of our monkeys which showed spontaneous ascorbic acid deficiency and in 2 other animals which were made ascorbic acid deficient by removing the vitamin from their diet.

3. Following the administration of the pure ascorbic acid, there was a normal course of parasitemia in one of the monkeys spontaneously ascorbic acid deficient and in those made ascorbic acid deficient by dietary means.

Withholding ascorbic acid therapy for more than 7 days appeared to give the spontaneously deficient monkeys time to produce an immunity and to control the infections. If ascorbic acid treatment was withheld from the animals made vitamin C deficient there was a prolongation of the course of infection but eventually the parasitemia overwhelmed the animal.

15585

Toxicity and Therapeutic Activity of Tyrothricin by Oral Administration.

JOHN A. KOLMER AND ANNA M. RULE.
From the Research Institute of Cutaneous Medicine, Philadelphia, Pa.

Since penicillin by oral administration has been found effective in the prophylaxis and treatment of certain infections, the matter of the toxicity and possible therapeutic effectiveness of tyrothricin by this route of administration is not without interest in relation to antibiotic therapy.

In these experiments a 2% alcoholic solu-

TABLE I.
Toxicity of Tyrothricin for Mice by Oral Administration.

No.	Dose per mouse* (g)	Survival: days 1	2	3	4	5	6	7	8	9	10
1	0.0004	—	—	—	—	—	—	—	—	—	—
2	0.0004	—	—	—	—	—	—	—	—	—	—
3	0.0006	—	—	—	—	—	—	—	—	—	—
4	0.0006	—	—	—	—	—	—	—	—	—	—
5	0.0008	—	—	—	—	—	—	—	D		
6	0.0008	—	—	—	—	—	—	—	—	D	
7	0.001	—	—	—	—	—	—	—	—	—	—
8	0.001	—	—	—	D						
9	0.0012	—	—	D							
10	0.0012	—	—	—	D						

* Administered orally 3 times a day.

TABLE II.
Therapeutic Activity of Tyrothrycin by Oral Administration to Mice.

Infections	Dose per mouse* (g)	No. mice	Survivals: days 1	2	3	4	5	6	7	8	9	10
Strept. hemolyt.	0.0004	10	10	10	9	5	3	2	2	0	0	0
,, ,,	controls	10	10	7	4	2	0	0	0	0	0	0
Pneu. (type I)	0.0004	12	12	10	8	8	6	4	4	4	4	4
,, ,, ,,	controls	12	12	7	2	0	0	0	0	0	0	0
,, ,, II)	0.0004	10	10	9	6	4	3	2	2	2	2	2
,, ,, ,,	controls	10	10	4	2	1	0	0	0	0	0	0
,, ,, III)	0.0004	10	10	4	2	2	0	0	0	0	0	0
,, ,, ,,	controls	10	10	3	0	0	0	0	0	0	0	0

* At 10 A.M., 1 P.M., and 4 P.M. daily.

tion of tyrothricin (Parke-Davis Co.) was employed, freshly prepared suspensions being prepared with sterile distilled water.

For toxicity tests 1:10 suspensions were administered containing 0.002 g per cc. Adult white mice were given 3 doses per day at 10 a.m., 1 p.m. and 4 p.m. by stomach tube in varying amounts, shown in Table I, for 10 days in succession. It will be observed that the maximum tolerated dose of the tyrothricin employed was about 0.0008 g per mouse.

Therapeutic tests were conducted with mice inoculated intraabdominally with 100 minimal lethal doses of 18-hour hormone broth cultures of *Streptococcus hemolyticus* (group A) and pneumococci of types I, II and III. Tyrothricin was administered by stomach tube in dose of 0.2 cc of 1:10 suspension (0.0004 g per mouse) 2 hours later with a second dose, 3 hours thereafter. Subsequent doses were administered at 10 a.m., 1 p.m. and 4 p.m. daily for 9 days in succession, covering a treatment period of 10 days. Untreated controls were included inoculated with the 4 cultures respectively. Heart blood cultures were made of the majority of succumbing mice and all were positive.

As shown in Table II, all of 10 mice infected with *Streptococcus hemolyticus* and treated with tyrothricin succumbed although 5 survived 1 to 3 days longer than the 10 untreated controls. Of 12 mice infected with type I pneumococcus and treated with tyrothricin 4 survived whereas all of 12 untreated controls succumbed within 4 days. Of 10 mice infected with type II pneumococcus and treated with tyrothricin 2 survived whereas all of 10 untreated controls succumbed within 5 days. All of 10 mice infected with type III pneumococcus and treated with tyrothricin succumbed although 2 survived for 2 days longer than 10 untreated controls.

In addition, 8 adult rabbits were inoculated intratesticularly with the Nichols-Hough strain of *T. pallidum*. In from 4 to 6 weeks all developed acute syphilomas with positive darkfield examinations. Two rabbits were kept as untreated controls while the remaining 6 were treated with tyrothricin administered by stomach tube once a day for 15 days in succession. Freshly prepared 1:100 suspensions of the compound in sterile distilled water were employed. The daily doses were 1 cc (0.0002 g), 5 cc (0.001 g) and 10 cc (0.002 g) per kg for each of 2 rabbits receiving the 3 different amounts. One of the 2 rabbits receiving 10 cc (0.002 g) died on the 8th day of treatment, apparently from the toxic effects of the compound. Clinical records and darkfield examinations were made at daily intervals. During and at the end of the period of treatment none of the animals showed any clinical evidences of healing of the testicular lesions as compared with the untreated controls and all showed persistently positive darkfield examinations. Under the circumstances lymph node transfers were regarded unnecessary.

Summary. The maximum tolerated dose of tyrothricin for adult white mice, administered by stomach tube 3 times a day for 10 days in succession, was about 0.0008 g per mouse. By this route of administration 3 doses of tyrothricin daily in dose of 0.0004 g per mouse were very slightly effective in the treatment of mice inoculated with virulent *Streptococcus hemolyticus* (group A) and type III pneumococcus, while definitely more effective in the treatment of mice inoculated with virulent types I and II pneumococci and especially type I. Tyrothricin in daily doses of 0.0002, 0.001 and 0.002 g per kg, administered by stomach tube for 15 days in succession, was completely ineffective in the treatment of acute testicular syphilis of rabbits.

15586 P

Inhibition of Bacterial Growth by Auxins.

RENE J. DUBOS.

From the Laboratories of the Rockefeller Institute for Medical Research, New York City.

We have observed that a number of synthetic unsaturated ring-containing acids endowed with auxin (plant hormone) activity can exert a bacteriostatic effect on the growth of certain bacterial species. The following substances were selected as representatives of different chemical types of auxins: indole-3-acetic acid, β-(indole-3)-propionic acid, -(indole-3)-n-butyric acid, α-naphthalene-acetic acid, β-naphthoxyacetic acid, 2,4-dichlorophenoxyacetic acid, 2,3,5-triiodobenzoic acid.[1]

The order of activity of these different substances on a few bacterial species is illustrated in Table I in which are given the concentrations of auxins required to cause a 50% inhibition of growth in a medium containing by weight 0.1% enzymatic hydrolysate of casein and 0.02% yeast extract. It should be emphasized that the inhibitory concentrations recorded in Table I have only a relative value since, as will be indicated later, tryptophane and peptone can antagonize the bacteriostatic action of auxins. It is also worth recording that the antibacterial activity of 2 of these substances had been recognized before anything was known of their plant hormone activity. Thus, it was known that indole acids (indole acrylic acid in particular) inhibit the growth of certain Gram-negative bacilli in synthetic media, and that this bacteriostatic effect can be reversed by tryptophane;[2] the growth of mycobacteria in synthetic Long's medium has been shown to be inhibited by 2,3,5-triiodobenzoic acid although the same substance stimulates oxygen uptake of these organisms

---

[1] Zimmerman, P. W., *Indus. and Eng. Chem.*, 1943, **35**, 596.

[2] Fildes, P., *Brit. J. Exp. Path.*, 1941, **22**, 20.

TABLE I.

Comparative Bacteriostatic Activities of Auxins Against Different Bacterial Species in Casein Hydrolysate-Yeast Extract Medium at pH 6.0.

	Approximate concentration of auxin (in %) required for 50% inhibition of growth*						
Inoculum 10-4 cc culture per cc medium	Indole acetic acid	Indole propionic acid	Indole butyric acid	Naphthalene acetic acid	Naphthoxy acetic acid	2-4 dichloro-phenoxyacetic acid	Triiodo-benzoic acid
Mycobacterium tuberculosis (human strain)	.01	.0005	.01	.005	.003	.003	.002
Streptococcus hemolyticus (H69D)	.01	.015	—	.01	.01	.015	.003
" *salivarius* (MG)	.001	—	—	.01	—	—	.001
Shigella paradysenteriæ (Sonne)	.003	.008	.01	.01	.01	.01	.01

— = not done.

* Readings of growth were made after incubation at 37°C for 1 day (streptococci and dysentery bacilli) or 10 days (tubercle bacilli). In general, growth did not increase significantly upon further incubation.

Concentrations of auxins 3 to 5 times greater than shown in the table are required for complete inhibition of growth.

when their metabolism is studied by the conventional Warburg technic.[3]

As shown in Table I the bacteriostatic activity of the different auxins varies greatly from one bacterial species to another, each substance appearing to exhibit a selective inhibitory effect against one or several groups of organisms. With the strains and preparations used in our tests, indole-3-acetic acid is the most active substance against the Gram-negative bacilli, whereas mycobacteria are peculiarly susceptible to β-(indole-3)-propionic acid; triiodobenzoic acid exhibits highest activity against certain strains of streptococci.

The inhibitory concentration of auxin is not markedly affected by the size of the inoculum (the inhibitory concentration increases 2- to 4-fold as the inoculum is increased 100- to 1000-fold). It is also independent of the addition of serum albumin to the medium (0.5% was the highest concentration of albumin tested). Peptone and tryptophane can reverse in part or completely the bacteriostatic effect of auxins, tryptophane being by far the most active in this respect. Reversal of bacteriostasis usually requires concentrations of tryptophane approximately 10 times higher than those of auxin added to the medium.

In the case of most organisms, the bacteriostatic activity of all substances increases as the medium becomes more acidic: in fact, it is often difficult to detect any inhibition of growth at slightly alkaline reactions. On the other hand, inhibition of growth of tubercle bacilli by auxins appears to be almost independent of the reaction of the medium.

When the concentration of auxin in the medium is insufficient to produce complete inhibition of growth, bacterial multiplication proceeds at first at a rate similar to that in the untreated culture. However, the presence of auxin results in an interruption of multiplication before maximal development has occurred; the density of the final culture is inversely related to the concentration of auxin. In other words, the antibacterial mechanism under consideration affects the total amount of bacterial protoplasm synthesized rather than the rate of growth of the culture.

---

[3] Saz, A. K., and Bernheim, F., *J. Pharm. and Exp. Ther.*, 1941, **73**, 78; Saz, A. K., Johnston, F. R., Burger, A., and Bernheim, F., *Am. Rev. Tub.*, 1943, **48**, 40.

15587

Chemotherapy of Certain Iodonium and Sulfonium Compounds.*

B. L. FREEDLANDER AND F. FRENCH.

From the Harold Brunn Research Institute, Mt. Zion Hospital, San Francisco, Calif.

In the following experiments diphenyliodonium chloride and several of its derivatives were tested for bacteriostatic and fungistatic action *in vitro* upon the following organisms: *M. tuberculosis, Staphylococcus aureus, E. coli, Proteus vulgaris, Streptococcus pyogenes, Ps. pyocyaneus, Cl. histolyticum,* and Monilia *(Candida krusei)*. Methyl diphenylsulfonium nitrate was also evaluated *in vitro*. In addition, several of the diphenyliodonium derivatives were investigated *in vivo* for their therapeutic action on tuberculous animals.

The sulfonium and iodonium compounds discussed in this paper are members of a class of compounds having large positive ions as an integral part of their structure. This class includes many organic phosphonium, arsonium and ammonium compounds and many derivatives of elements on the right-hand side of the periodic table. Certain compounds in this group are powerful cationic detergents, for example the aryl or aralkyl-alkyl-dimethyl-ammonium salt, and these compounds are generally and actively bacteriostatic by virtue of their surface-active character. The compounds discussed in this paper, however, are not notably surface active but do show strong and selective biostatic actions.

Methods. In Vitro Experiments. For the *in vitro* studies the tubercle bacilli were grown on Proskauer and Beck synthetic media, pH 7.0. The pyogenic bacteria were grown on beef heart broth, pH 7.2. *Cl. histolyticum* was grown in Brewer's fluid thioglycollate medium and in standard beef heart broth containing 0.5% glucose. For the culture of the fungus, Monilia, Sabouraud's agar was used. The chemical compounds were dissolved in water to make 0.1% solutions; subsequent dilutions were made directly into the media without further heating. Dilutions into agar were made at approximately 60°C. Two strains of tubercle bacilli were used, the virulent H37Rv and the avirulent strain No. 607. The media were inoculated with one loopful of fresh culture of tubercle bacilli. The beef heart broth was inoculated with 0.1 cc of a 24-hour culture of the pyogenic bacteria. For the fungistatic tests, the agar slants were streaked with one loopful of a 3-day culture of Monilia. All the cultures were incubated at 37°C. The cultures were read at the following periods of time: virulent tubercle bacilli—3 weeks; avirulent tubercle bacilli—4 days; pyogenic bacteria—1 day and Monilia—3 days. The highest bacteriostatic or fungistatic dilution was taken as that dilution in which there was no or very slight growth.

Animal Experiments. Nine guinea pigs, averaging 350 g in weight, were used to evaluate each compound for its therapeutic effect in animals infected with tuberculosis. The animals were inoculated in the left

---

* The present report is part of a cooperative investigation on tuberculosis, and has been supported by funds provided by the Committee on Medical Research of the National Tuberculosis Association, the California Tuberculosis Association, the San Francisco Tuberculosis Association, the Columbia Foundation of San Francisco, and the Rose Lampert Graff Foundation of Beverly Hills, California.

groin with 0.01 mg of H37Rv tubercle bacilli. The drugs were administered either orally or hypodermically beginning on the day of infection. For oral administration the drug was dissolved in water and then mixed with ground rabbit pellets. For hypodermic administration the drugs were ground with a 1.5% beeswax-sesame oil mixture, and the resultant mixtures were injected in 0.3 cc amounts subcutaneously. All experiments were terminated at the end of 6 weeks. Pathology was evaluated numerically by the method of Sweany, Sher and Kloeck,[1] in which the maximum gross involvements of the organs were given the following numerical ratings: lymph nodes 8, spleen 24, liver 28, and lungs 40. Hemoglobin determinations were made at the end of the experiments.

Results. The *in vitro* bacteriostatic results are recorded in Table I. The parent compound, diphenyliodonium chloride, inhibited the growth of tubercle bacilli in very high dilutions; the virulent culture in a dilution of 1:800,000 and the avirulent strain in a concentration of 1:600,000. In the presence of 50% horse serum, diphenyliodonium chloride showed no decrease in bacteriostatic effect (1:600,000). The di-p-methyl derivative, di-p-tolyliodonium iodide, was equally effective. The di-p-amino derivative, di-p-aminodiphenyliodonium iodide, was somewhat less bacteriostatic. Acetylation of the amino groups decreased the tuberculostatic action considerably. (o)-Carboxydiphenyliodonium iodide was without inhibiting action. Methyl-diphenylsulfonium nitrate was also without tuberculostatic action. *p*-Aminobenzoic acid did not antagonize the inhibition of tubercle bacilli by diphenyliodonium chloride.

Diphenyliodonium chloride showed a moderate bacteriostatic action on *Streptococcus pyogenes*, being effective in a dilution of 1:80,000. This compound also inhibited the growth of *Staphylococcus aureus* in a concentration of 1:40,000. Diphenyliodonium chloride exhibited still less inhibiting effect

TABLE I.

Highest Bacteriostatic or Fungistatic Dilution *in Vitro.* (× 10,000.)

	Tubercle bacillus H37Rv	Tubercle bacillus No. 607	*Staphylococcus aureus*	*E. coli*	*Proteus vulgaris*	*Streptococcus pyogenes*	*Ps. pyocyaneus*	*Cl. histolyticum*	*Monilia krusei*
Diphenyliodonium chloride $(\langle\rangle\ \text{I}\ \langle\rangle)^{+}\ \text{Cl}^{-}$	80	60	4	1	2	8	<1	20	40
Diphenyliodonium iodide		60	2	<1	2				
Di-p-tolyliodonium iodide		60	2	<1	1				
Di-p-acetylaminodiphenyliodonium iodide		2	2	**<1**	<1				
Di-p-aminodiphenyliodonium iodide		10	<1	<1	<1				
o-Carboxydiphenyliodonium iodide		<1	<1	<1	<1				
Methyldiphenylsulfonium nitrate		1	2	1	<1	<1	<1		10

Controls with potassium iodide were run to exclude the effect of ionic iodine; these were negative for bacteriostatic action. Phenol showed negligible fungistatic action on Monilia (<1:10,000).

---

[1] Sweany, H. C., Sher, B. C., and Kloeck, J. M., *Am. Rev. Tuberc.*, 1946, **53**, 254.

TABLE II.
Summary of Therapeutic Efficacy in Tuberculous Animals.

Group	Compound	Dosage	Avg Path. Rating	Avg wt Gain	Hglb.
A	Diphenyliodonium chloride	8 mg daily oral	40.6	172	11.8
	Controls		49	223	10.9
B	Diphenyliodonium chloride	1 mg daily* hypo.	38.1	155	11.1
	Controls		46.4	101	10.8
C	Di-p-acetylaminodiphenyliodonium iodide	1 mg every other day hypo.	46.6	158	12.2
	o-Carboxyldiphenyliodonium iodide	2 mg every other day hypo.	42	161	11.9
	Controls		39.3	215	12.4

* After 3 weeks of therapy the drug was given only on alternate days.

on *P. vulgaris*, *E. coli* and *Ps. pyocyaneus*. On the other hand, it showed a pronounced inhibiting action on Monilia (1:400,000). None of the derivatives of this compound was significantly effective in inhibiting the growth of *Staphylococcus aureus*, *B. coli*, or *B. proteus*. Likewise the compound, methyldiphenylsulfonium nitrate, was ineffective against these organisms; however, this compound showed an excellent inhibiting action on Monilia.

Cl. histolyticum grown in the beef heart medium was inhibited by diphenyliodonium chloride in a dilution of 1:200,000. In the thioglycollate medium, however, a dilution of 1:10,000 was required for inhibition. The addition of concentrations of thioglycerol ranging from 1:6,000 to 1:120,000 to Proskauer-Beck medium had no significant effect on the inhibition of tubercle bacilli by diphenyliodonium chloride. The replacement of thioglycerol by cysteine or sodium thioglycollate also yielded negative results.

The *in vivo* results are summarized in Table II. Diphenyliodonium chloride and several of its derivatives were administered therapeutically to guinea pigs infected with tubercle bacilli. On oral administration the animals tolerated 8 mg daily of the compound, diphenyliodonium chloride, without signs of toxicity. However, only 1 mg could be given hypodermically and only 70% of the animals survived this therapy. None of the compounds showed any significant therapeutic action.

Discussion. The very large difference between the activities of the iodonium and sulfonium salts may be, in part, due to gross geometrical differences. However, more specific factors such as ionic radius, polarizability, and oxidizing power are probably more significant. For the iodonium compounds certain configurational factors of importance have been demonstrated. For example, in (o)-carboxydiphenyliodonium iodide there is an enormous drop in tuberculostatic activity. This may be due to the formation of a compact inner salt structure at neutral or alkaline pH's. Such a structure could effectively block the manifestation of the bacteriostatic activity of the iodonium group. The drop of activity in p,p′diaminodiphenyliodonium iodide may be due to the electron donor character of the p-amino groups tending to decrease the positive character of the iodonium groups. Alternatively, amino groups and especially carboxyl groups may block entry of the molecule to the site of the characteristic toxic action of the iodonium group.

As to the mechanism of action of the iodonium compounds the following observations may be cited. It has been noted by Sandin and others[2] that the stability of aryliodonium salts decreases with increasing polarizability of the anion. For example, di-o-tolyliodonium bromide decomposes at 178°C, the iodide at 155°C, and the sulfide at room temperature. The less stable onium compounds of other classes show similar behavior. Snyder and Speck[3] have shown that in hot solutions containing benzylphenyldimethylammonium ion, cleavage of the benzyl group is easily effected by HSO_3^-, $SO_3^=$, HS^-, $S^=$, $S_2O_3^=$ and CNS^- while

---

[2] Sandin, R. B., *Chem. Rev.*, 1943, **32**, 249.

[3] Snyder, H. R., and Speck, J. C., *J. Am. Chem. Soc.*, 1939, **61**, 668.

CN^-, OH^-, $OC_6H_5^-$, and $PO_4^{\equiv}$ are ineffective. The released benzyl radicals arylate the anions. In a microorganism, if such arylation occurs, a free and functionally active mercapto group, essential to the metabolism of the organism, would be converted into a nonactive thioether group.

Conclusions. It may be suggested, tentatively, that the activity of iodonium compounds may, in some cases, be due to interaction with certain mercapto groups essential to the microorganisms. This formulation rests largely on observations of the high reactivity of iodonium compounds toward sulfide, hydrosulfide and similar ions in simple chemical systems. That the detail of the mechanism of inhibition is different for *Cl. histolyticum* and *M. tuberculosis* is apparent from the experiments involving thioglycollate. In the case of *M. tuberculosis* we have not found a mercapto compound which antagonizes the effect of the iodonium compounds. That both sulfonium and iodonium compounds inhibit Monilia, while the iodonium compounds alone inhibit the growth of tubercle bacilli, suggest that a similar mechanism of action may be involved with both compounds for Monilia, but not in the case of *M. tuberculosis*.

15588

Effect of Some Barbiturates on Tissues *In vitro.*

C. M. POMERAT, G. A. DRAGER, AND J. T. PAINTER.

From the Department of Anatomy and of Neuro-Psychiatry, Medical Branch of the University of Texas, Galveston, Texas.

It is well known to workers with tissue cultures that outgrowths from explants of brain, ganglia and spinal cord can be easily obtained in fluid or semisolid media even without the addition of embryonic extract. The cultivation of spinal ganglia has proved to be a valuable test material in a variety of experimental studies (Burt,[1,2] Meyer and Jablonski,[3] Murnaghan,[4] Weiss,[5-7] Weiss and Wang[8,9]). Relatively little use has been made of nerve cord cultures even though the technic for obtaining such preparations is considerably easier than that required for the dissection of ganglia.

The procedure for cord cultures which has been used in our laboratories is schematized in Fig. 1 to 4. The necks of embryos of suitable ages are cut (Fig. 1) at their base with scissors or knives. The spinal cord is expelled from the neural canal by compressing the tissue in the mid-dorsal line with 2 pairs of forceps moved alternately in the direction of the sectioned vertebral column. Thus a cervical (Fig. 2) and a thoracolumbar segment (Fig. 3) of cord can be obtained. These are washed in Tyrode's solution and then transferred to a ground glass slide and cut with sharp knives. For this purpose we have found fragments of wafer razor blades soldered in the eye of a needle more satisfactory than cataract knives (Fig. 4a). Transverse sections varying from ¼ to ½ mm can be cut easily by moving knife tips, placed at right angles to the cord segment, in opposite directions (Fig. 4 and 4a). After washing in Tyrode's solution hang-

---

[1] Burt, A., *J. Cell. and Comp. Physiol.*, 1943, **22**, 145.

[2] Burt, A., *J. Cell. and Comp. Physiol.*, 1943, **22**, 205.

[3] Meyer, H., and Jablonski, W., *J. Anat.*, 1937, **72**, 62.

[4] Murnaghan, D. P., *Anat. Rec.*, 1941, **81**, 183.

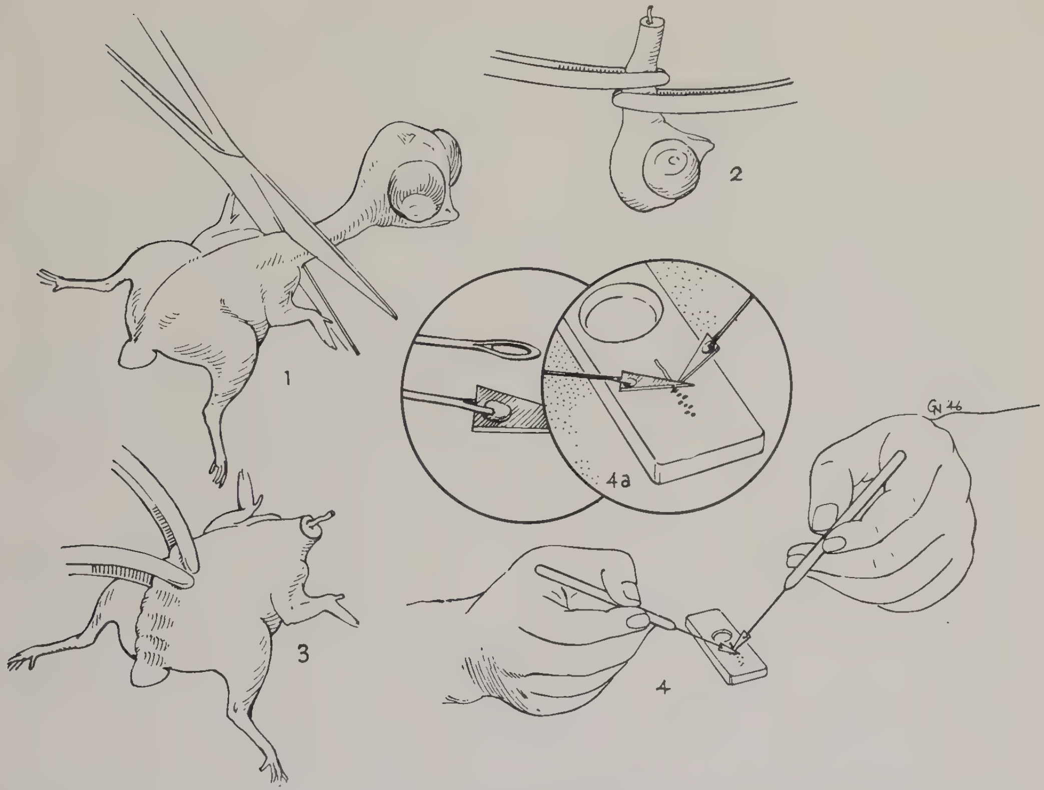
[5] Weiss, P., *J. Exp. Zool.*, 1934, **68**, 393.

[6] Weiss, P., *Anat. Rec.*, 1944, **88**, 205.

[7] Weiss, P., *J. Exp. Zool.*, 1945, **100**, 353.

[8] Weiss, P., and Wang, H., *Anat. Rec.*, 1936, **67**, 105.

[9] Weiss, P., and Wang, H., PROC. SOC. EXP. BIOL. AND MED., 1945, **58**, 273.



ing drop preparations were made by placing cord sections in a drop of 25% embryonic extract from chicks incubated for 6-10 days with or without the inclusion of test solutions. A drop of 50% rooster plasma was then mixed thoroughly into the medium.

A detailed description of the morphological aspects of the resulting outgrowth as a function of the age of the cord and other variables will be reported in a separate study. The conditions found most suitable for the purpose of the present report consisted of using cords from chicks incubated for 9 days. Heart fragments from the same individuals and cultivated for 72 hours were used for comparison of growth in nonnervous tissue. The medium was measured out as uniform-sized drops from No. 27 needles mounted onto sterile 1 cc tuberculin syringes. Living cultures were observed after 24 and 48 hours of incubation and were then fixed. Bouin's solution was found to be superior to a variety of other fixatives as a preparation for staining with Bodian's technic.

TABLE I.
Outgrowth from Spinal Cord After 48 Hours and Heart After 72 hours. 9-day Chick Embryos.

Conc.	Seconal sodium		Pentobarb. sodium		Sodium amytal	
	Cord	Heart	Cord	Heart	Cord	Heart
1:800	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0
1:1600	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0
1:3200	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	½ ½ 1 0
1:6400	0 0 0 0	1 ½ 1 1	1 ½ 1 1	2 2 3 2	2 1 1 2	1 1 2 1
1:8000	1 0 ½ 1	2 2 2 2	2 1 2 2	3 2 4 3	3 3 3 2	2 2 2 1
1:12800	1 2 2 1	3 3 3 3	2 1 2 0	3 3 2 3	—	—

Outgrowth of 1.6 mm = 4+; 1.2 = 3+; 0.8 = 2+; 0.4 =1+; a few short fibers = ±.

Approximately 700 cultures were prepared to test the effect of Seconal Sodium, Pentobarbital Sodium, Sodium Amytal and 14 barbiturate compounds supplied by Dr. K. K. Chen. The authors are deeply indebted to The Lilly Research Laboratories for their kind assistance and support of this study.

Using an ocular micrometer it was possible to measure the amount of outgrowth in living cultures. At the end of 48 hours the maximum length of fibers in control cultures was found to be approximately 1.6 mm. This value served in a scale for grading growth in experimental cultures as follows: outgrowth of 1.6 mm = 4+; 1.2 = 3+; 0.8 = 2+; 0.4 = 1+; a few short fibers = ±. As a practical tool for an extensive survey of a large number of drugs at various concentrations, this system of tabulation was found too cumbersome and effort was directed simply toward finding the minimum concentration of a test solution which would first prevent all outgrowth of fibers and, in the case of heart fragments, of all outwandering cells. After some preliminary study test concentrations for the various barbiturates were prepared at final concentrations of 1:800, 1:1600, 1:3200, 1:6400, 1:8000, 1:12,800 and 1:16,000. Four cultures were set up for each tissue and test solution. All component materials for a given experiment were constant except the barbiturate factor. Each experiment was repeated and the dosage was overlapped several times so that the whole series of results were comparable. Tyrode's solution was used in the preparation of all barbiturate concentrations.

Table I shows that total inhibition of fiber outgrowth was obtained at 1:6400 for Seconal Sodium and 1:3200 for both Pentobarbital Sodium and Sodium Amytal. Outgrowth from heart fragments seemed somewhat more resistant to the deleterious effects of the barbiturates.

The other barbituric acid derivatives fall into 2 groups, as listed in Table II. Compounds 1 to 8 are convulsants and compounds 9 to 14, hypnotics. The median convulsive dose (CD_{50}), median lethal dose (LD_{50}), and median anesthetic dose (AD_{50}) have

TABLE II. Convulsive (CD), Lethal (LD), and Anesthetic Dose (AD) of Barbiturates in Relation to Outgrowths from Chick Spinal Cord and Heart Fragments *in Vitro*.

Compounds	Mol. wt Na salt	Route	CD_{50} ± S.E. (Rats) mg/kg	LD_{50} ± S.E. (Rats) mg/kg	Inhibition Cords, 48°	Inhibition Hearts, 72°
1. 1,3-dimethyl butyl allyl barbituric acid	284	i.v.	—	50.0 ± 3.02	1:6400	1:1600
2. Sodium 3,3-dimethyl allyl ethyl thiobarbiturate	263	,,	12.1 ± 0.95	53.1 ± 2.42	1:1600	1:800
3. 3,3-dimethyl allyl allyl barbituric acid	268	,,	8.89 ± 0.52	16.7 ± 0.45	1:3200	1:800
4. Sodium N-methyl-3,3-dimethyl allyl ethyl barbiturate	261	,,	1.39 ± 0.21	4.29 ± 0.48	1:1600	1:800
5. Sodium isobutyl 1-methyl allyl thiobarbiturate	277	,,	3.9 ± 0.39	21.57 ± 1.66	*1:800	1:800
6. 1,3-dimethyl butyl ethyl barbituric acid	272	i.p.	9.74 ± 0.36	18.13 ± 0.61	1:3200	1:800
7. 3,3-dimethyl allyl allyl thiobarbituric acid	284	i.v.	8.92 ± 0.535	94.08 ± 8.47	1:12800	1:6400
8. 3,3-dimethyl allyl ethyl barbituric acid	256	,,	5.28 ± 0.31	8.84 ± 0.32	*1:800	*1:800
			AD_{50} ± S.E. (Rats) mg/kg			
9. n-propyl-2-methyl allyl barbituric acid	256	i.p.	114.7 ± 3.7	265.1 ± 7.2	*1:800	1:800
10. sec-butyl-2-methyl allyl barbituric acid	270	,,	76.3 ± 3.1	184.8 ± 3.4	1:800	1:800
11. Allyl-1-methyl allyl barbituric acid	254	,,	69.01 ± 2.76	135.6 ± 6.23	1:800	*1:800
12. Ethyl-3-methyl allyl barbituric acid	242	,,	88.43 ± 6.7	229.7 ± 5.10	*1:800	*1:800
13. n-propyl-1-methyl allyl barbituric acid	256	,,	76.52 ± 2.66	227.5 ± 5.5	*1:800	*1:800
14. iso-propyl-3-methyl allyl barbituric acid	256	,,	66.1 ± 3.4	168.3 ± 7.5	*1:800	*1:800

* Subtotal inhibition.

been determined in The Lilly Research Laboratories. It is obvious that out of the whole series the hypnotic barbiturates are less toxic in whole animals than the convulsant barbiturates. Our results indicate that there is some correlation in tissue culture. For example, compounds 9 to 14 which have a lower toxicity *in vivo* were all found to inhibit cellular outgrowth at 1:800 or a little less. Compounds 1 to 8 which are more toxic *in vivo* were found similarly more damaging to tissues *in vitro*. In the latter group, notable exceptions were observed. Thus, although the LD_{50} of compound 8 was only 8.84 ± 0.32 mg/kg in rats, nerve outgrowth from cord and migration of cells from heart fragments was not completely inhibited at 1:800. Compound 5 was also characterized by a high toxicity *in vivo* but by a low toxicity *in vitro*. Another unpredictable effect was found for 3,3-dimethyl allyl allyl thiobarbituric acid (No. 7), which had an LD_{50} of 94.08 ± 8.47 mg/kg, but which proved to be the most toxic member of the series in terms of outgrowth of living tissue. No fibers were observed on repeated tests from cords—in a medium containing 1:12,800 of this compound. Heart cells showed no migration at 1:6400. It may be concluded, therefore, that while there is a general correspondence between the toxicity of barbiturates *in vivo* and the presence of some especially vulnerable tissue of the whole organism would modify results obtained in tissue cultures. Cyto-pharmacological work of this type (*e.g.* Jacoby, Medawar and Willmer,[10] Pomerat,[11] Pomerat, Capouya and Greenleaf,[12] Pomerat, Lay and Emerson,[13] Salle[14]) agree in demonstrating that tissue cultures offer a stringent test of toxic effects which may ultimately serve as an aid in exploring possible mechanisms of action.

These studies suggest that it is not enough, in appraising possible drug toxicity, to be content merely with estimations of gross lethal effects. It may also be helpful to survey drug toxicity with respect to particular organs and tissues under appropriate conditions.

Summary. Hanging drop tissue cultures of spinal cord from chicks incubated for 9 days were used to test the inhibitory action of various barbiturates on the outgrowth of nerve fibers. Using concentrations of 1:800, 1:1600, 1:3200, 1:6400, 1:8000 and 1:12,800, total inhibition was obtained at 1:6400 for Seconal Sodium, and at 1:3200 for both Pentobarbital Sodium and Sodium Amytal. The convulsive, lethal and anaesthetic dose of 14 other barbiturate compounds was generally well correlated with *in vitro* estimates of toxicity. However, some notable exceptions were found. For example, the lethal dose of 3,3-dimethyl allyl ethyl barbituric acid was 8.84 ± 0.32 mg/kg; and the second most damaging compound tested *in vivo*, but belonged to the least toxic group tested *in vitro*. In contrast, 3,3-dimethyl allyl allyl thiobarbituric acid which had a lethal dose of 94.08 ± 8.47, proved to be the most toxic member of the entire series in terms of outgrowth of living tissue. Corresponding cultures using fibroblast outgrowth from chick heart fragments generally proved somewhat more resistant to barbiturates than fiber outgrowths from cord explants. These results suggest that the survey of drug toxicity should include studies on particular tissues in addition to estimates of gross lethal effects.

---

[10] Jacoby, F., Medawar, P. E., and Willmer, E. N., *Brit. M. J.*, 1941, **2**, 149.

[11] Pomerat, C. M., PROC. SOC. EXP. BIOL. AND MED., 1942, **41**, 345.

[12] Pomerat, C. M., Capouya, M., and Greenleaf, F. I., *Tex. Rep. on Biol. and Med.*, 1944, **2**, 97.

[13] Pomerat, C. M., Lay, M., and Emerson, G. A., *Tex. Rep. on Biol. and Med.*, 1944, **2**, 316.

[14] Salle, A. J., PROC. SOC. EXP. BIOL. AND MED., 1944, **56**, 151.

15589 P

Influence of Sex on Resistance of Albino Rat to Intraperitoneal Inoculation of *Brucella abortus*.[*]

ALBERTO ASCOLI. (Introduced by C. G. Tedeschi).
From the Agricultural Experiment Station, New Brunswick, N.J.

In the course of experiments (August 1944-August 1946) by Dr. Herbert J. Metzger and the author, on the suggestion of Dr. Robert A. Cooke, testing various sera for protective power against *Brucella abortus,* I came across a fact hitherto unnoticed which is reported in the present note.

Material and Methods. Sera drawn from normal cattle, from animals immunized with Strain 19, and from cows affected with Bang's disease were used. Albino rats (weighing from 100 to 140 g) of both sexes were inoculated intraperitoneally with small amounts (less than 1 ml) of serum and a few hours later with a lethal dose of virulent Brucella organisms cultured for 48 hours on tryptose agar. The animals which did not die were killed in a few days by inhalation of chloroform. A thorough inspection of the abdominal and thoracic organs and glands was made in all animals, and a bacteriological examination of the blood was carried out routinely. Bacteriological examinations were also made of any tissues showing lesions which seemed to be caused by other pathogens, and the causative agent isolated.

Observations. It is observed that under comparable experimental conditions on the average the female rats died earlier from the infection than the males. A retrospective scrutiny made of the postmortems, including the control rats, with respect to sex of 141 inoculated rats revealed that of the 81 males, 28 (34.6%) survived and 53 (65.4%) succumbed on the average 3.6 days after inoculation. Of the females, given the same *Brucella abortus* suspension, only 5 survived (8.3%), while 55 (91.7%) died after an average life span of 2 days. In later experiments the sex was recorded for each rat and the lower resistance of female rats to intraperitoneal inoculation of *Brucella abortus* was confirmed directly in tests on more than 2000 rats. It was found that the M.L.D. for female rats was approximately ½ to ⅓ that required for males. As to the influence of age, a few tests made on older female rats (weight 300-400 g) seemed to indicate that they are more susceptible than young female rats.

Comment. The reason for the greater susceptibility of the females has not been found, though all the inoculated rats were submitted to careful postmortem and bacteriological examination. The postmortem findings were manifold but practically the same in male and female rats.

Summary. Original experimental observations are reported pointing to the greater susceptibility of the female albino rat over the male albino rat to intraperitoneally-induced *Brucella abortus* infection. Sex had been previously disregarded in research work on Brucella infection of rats.

---

[*] This preliminary note is but a part of the main study on brucellosis supported by the Mennen Fund.

15590

Vitamin B Complex Studies with Diets Differing in the Level of Protein.

LEMUEL D. WRIGHT AND HELEN R. SKEGGS.

From the Nutritional Laboratories, Department of Pharmacology, Medical Research Division, Sharp and Dohme, Inc., Glenolden, Pa.

The level of dietary protein has been demonstrated by several investigators to be a factor in the metabolism of certain members of the vitamin B complex. The studies of Sarett and Perlzweig[1] and of Czaczkes and Guggenheim[2] have shown that on diets low in protein rats are incapable of retaining ingested riboflavin or maintaining tissue stores of this factor. The retention and storage of nicotinic acid similarly is influenced by the protein intake. Wright, Skeggs, and Sprague showed[3] that rats receiving liberal protein diets were less susceptible to the deficiency syndrome induced by feeding succinylsulfathiazole (SST) in *highly purified* diets. Such animals had higher hepatic stores of folic acid and pantothenic acid than control rats receiving the usual amount of protein in the diet. Nelson and Evans found[4] that pantothenic acid deficiency was less readily induced in rats receiving high protein diets than in those receiving diets of usual composition. The concentration of thiamine in the tissues of rats is not affected by the level of protein in the diet.[1] The requirement of rats for pyridoxine is increased when they are fed high protein diets.[5]

The studies reported in this paper were carried out for the purpose of obtaining additional information concerning the effect of the level of dietary protein on the metabolism of certain B vitamins in the rat. Diets used that were low in protein were, in consequence, deficient in essential amino acids. The coexistence of inadequate protein intakes and certain vitamin deficiencies frequently has been observed among various population groups.

Procedure. Three feeding experiments involving diets in which the level of protein was varied have been carried out. The low protein diet (5%) was compounded as follows: Casein, 50 g; sucrose, 748 g; salt mixture,[6] 40 g; cellus flour, 40 g; fat ('Primex'), 100 g; corn oil, 20 g; A-D-E concentrate,[3] 1 g; choline chloride, 1 g; vitamin K ('Menadione'), 10 mg; thiamine chloride, 8 mg; riboflavin, 16 mg; pyridoxine hydrochloride, 8 mg; nicotinic acid, 40 mg; calcium pantothenate, 44 mg; *p*-aminobenzoic acid, 40 mg; inositol, 216 mg. In the remaining diets, the protein level was increased, and succinylsulfathiazole was included at the expense of the sucrose. In the first experiment diets containing 5, 7, 10 and 18% casein and 18% casein plus 2% SST were fed *ad libitum* to male albino rats caged individually over wide mesh screening. Food intake determinations were carried out on representative rats throughout the course of the experiment. The second experiment was similar to the first except that controlled feeding was employed by arranging the rats in quintuplets and restricting the food intake of those receiving 7, 10 and 18% casein, and 18% casein plus 2% SST diets to that consumed by the animals receiving the 5% casein diet. The third experiment involved 3 groups of rats. The first received the 18% casein diet *ad libitum.* The second group was pair-fed with the first group and received the 30% casein diet. The third group received the 30% casein diet *ad libitum.* All animals were weighed at weekly intervals.

At intervals throughout the experiments 24-hour fecal collections were made. One-

---

[1] Sarett, H. P., and Perlzweig, W. A., *J. Nutrition*, 1943, **25**, 173.

[2] Czaczkes, J. W., and Guggenheim, K., *J. Biol. Chem.*, 1946, **162**, 267.

[3] Wright, L. D., Skeggs, H. R., and Sprague, K. L., *J. Nutrition*, 1945, **29**, 431.

[4] Nelson, M. M., and Evans, H. M., PROC. SOC. EXP. BIOL. AND MED., 1945, **60**, 319.

[5] Cerecedo, L. R., and Foy, J. R., *Arch. Biochem.*, 1944, **5**, 207.

[6] Hubbell, R. B., Mendel, L. B., and Wakeman, A. K., *J. Nutrition*, 1937, **14**, 273.

TABLE I.
Summary of Growth, Survival, and Food Intake Data.

Level of protein, %	Duration of exp., days	Change in wt, g	Survivors	Avg daily food intake, g
	Ad libitum feeding exper.			
5	76	—4	6/8	4.7 (50)*
7	76	25	7/8	5.7 (50)
10	76	83	8/8	9.3 (52)
18	76	173	8/8	12.1 (52)
18 + SST	76	89	2/8	8.4 (44)
	Controlled feeding exper.			
5	56	—5	8/8	4.8 (448)
7	56	1	7/8	4.8
10	56	11	8/8	4.8
18	56	24	7/8	4.8
18 + SST	56	8	5/8	4.8
	High protein feeding exper.			
18 *ad lib.*	35	89	4/4	10.2 (140)
30 controlled	35	86	4/4	10.2
30 *ad lib.*	35	101	4/4	9.9 (44)

* Figures in parentheses represent number of determinations performed.

half of each sample was digested with takadiastase in pH 4.5 acetate buffer[7] prior to microbiological determination of folic acid, pantothenic acid, riboflavin, and nicotinic acid. The other half of each sample was autoclaved with 6N H_2SO_4 for one hour prior to microbiological assay for biotin. As an inhibitor of SST 10 mg of para aminobenzoic acid was added to each 100 ml of medium used for microbiological assay.

At intervals throughout the first 2 experiments and at the conclusion of the 3 experiments total and differential white counts were made. Blood was obtained by clipping the tail except for that obtained at the conclusion of the experiments when it was obtained following decapitation of the rats. Separate experiments have demonstrated that white cell and differential counts obtained on blood by decapitation are in agreement with corresponding counts made on tail blood. Terminally red blood cell counts, hemoglobin, and hematocrit determinations were made.

Following decapitation of the rats the livers were dissected out and prepared for microbiological assay. One-half of each liver in a fragmented state was digested with takadiastase under water[8] prior to assay for folic acid, pantothenic acid, riboflavin and nicotinic acid. The other half of each sample was autoclaved with 6N H_2SO_4 for one hour[9] prior to microbiological assay for biotin.

Folic acid[10] and riboflavin[11] were determined microbiologically with *L. casei.* Biotin,[12] pantothenic acid,[13] and nicotinic acid[14] were determined with *L. arabinosus.*

Results. The growth, survival, and food intake data are summarized in Table I. In the *ad libitum* experiment alopecia, porphyrin-caked whiskers, and ophthalmitis were seen in most of the rats receiving the 5% casein and 18% casein plus SST diets. These deficiency signs were observed in a few of the rats receiving the 7% casein diet. The remaining animals were normal in appearance. Except for the smaller size of the rats restricted in food intake (Experiment 2) the appearance of the animals of the various groups was quite similar to that observed in the *ad libitum* experiment. The animals of the third experiment were normal in appearance.

---

[7] Cheldelin, V. H., Eppright, M. A., Snell, E. E., and Guirard, B. M., *The University of Texas Publication*, 1942, No. 4237, 15.

[8] Wright, L. D., Skeggs, H. R., and Welch, A. D., *Arch. Biochem.*, 1945, **6**, 15.

[9] Wright, L. D., and Welch, A. D., *J. Nutrition*, 1944, **27**, 55.

[10] Landy, M., and Dicken, D. M., *J. Lab. and Clin. Med.*, 1942, **27**, 1086.

[11] Snell, E. E., and Strong, F. M., *Ind. Eng. Chem., Anal. Ed.*, 1939, **11**, 346.

[12] Wright, L. D., and Skeggs, H. R., PROC. SOC. EXP. BIOL. AND MED., 1944, **56**, 95.

[13] Skeggs, H. R., and Wright, L. D., *J. Biol. Chem.*, 1944, **156**, 21.

[14] Snell, E. E., and Wright, L. D., *J. Biol. Chem.*, 1941, **139**, 675.

The fecal assay data (Table II) demonstrate that in *ad libitum* feeding the amount of folic acid and biotin synthesized in the intestine is a function of the level of dietary protein. Likewise the fecal elimination of pantothenic acid, riboflavin, and nicotinic acid, originating in part from the diet and in part from bacterial synthesis in the intestine, parallels the level of dietary protein. The data from the controlled feeding experiment demonstrate that the increased amounts of these factors eliminated on the higher levels of protein result only in part from increased food intakes accompanying the ingestion of diets more nearly adequate. With equal food intakes more biotin, pantothenic acid, and nicotinic acid are eliminated as the level of dietary protein is increased. The data with respect to folic acid and riboflavin were inconclusive. It should be pointed out that the fecal elimination of folic acid and biotin encountered in the first experiment in the 5% casein groups was as low as that found on the 18% casein plus SST diets. The daily elimination of pantothenic acid, riboflavin, and nicotinic acid was not significantly altered by the ingestion of SST.

The hepatic storage data, summarized in Table III, likewise demonstrate that in *ad libitum* feeding the storage of folic acid, biotin, pantothenic acid, riboflavin, and nicotinic acid, based on γ/g content of the liver, parallels the level of dietary protein. Here again when the food intakes were reduced to those consumed by the animals receiving the 5% casein diets the hepatic storage of the 5 factors paralleled the level of dietary protein. Similarly it should be pointed out that the hepatic stores of folic acid and biotin encountered in the 5% casein groups were as low as those found on the 18% casein plus SST diets and were characteristic of defi-

TABLE II. Summary of Fecal Assay Data.

Level of protein, %	No. of samples	Folic acid, γ/rat/day	Biotin, γ/rat/day	Pantothenic acid, γ/rat/day	Riboflavin, γ/rat/day	Nicotinic acid, γ/rat/day
			Ad libitum feeding experiment.			
5	5	1.5 (1.1 -1.6)*	0.20 (0.15-0.25)	10 (6-12)	4.4 (4.0- 5.1)	11 (8-13)
7	5	2.0 (1.7 -2.4)	0.30 (0.21-0.33)	12 (11-13)	5.7 (4.2- 6.4)	12 (11-12)
10	5	3.5 (2.5 -4.8)	0.35 (0.28-0.41)	20 (15-27)	8.4 (5.2- 9.4)	21 (15-23)
18	5	6.0 (5.0 -7.1)	0.61 (0.38-0.75)	28 (22-40)	11.4 (7.3-14.1)	33 (25-46)
18 + SST	5	1.5 (1.2 -1.9)	0.29 (0.26-0.33)	25 (17-29)	13.1 (9.0-17.1)	40 (27-46)
			Controlled feeding experiment.			
5	4	1.3 (1.1 -1.7)	0.13 (0.10-0.14)	7 (5- 9)	4.9 (3.0- 6.2)	8 (6-11)
7	4	1.2 (1.0 -1.4)	0.16 (0.15-0.20)	10 (6-17)	4.2 (2.8- 5.2)	8 (6-12)
10	4	1.3 (1.0 -1.6)	0.26 (0.22-0.30)	11 (7-17)	3.7 (3.1- 4.1)	9 (6-15)
18	4	1.4 (1.2 -1.6)	0.24 (0.21-0.30)	14 (12-16)	3.6 (3.0- 4.3)	12 (10-16)
18 + SST	3	0.53 (0.36-0.66)	0.18 (0.17-0.19)	11 (10-12)	4.0 (3.3- 4.6)	14 (12-16)
			High protein feeding experiment.			
18 *ad. lib.*	3	4.0 (3.3 -4.5)	1.4 (0.85-2.3)	22 (18-26)	7.1 (6.0- 8.0)	27 (23-32)
30 controlled	3	3.8 (3.7 -3.9)	1.1 (0.92-1.4)	28 (24-32)	8.1 (6.9- 9.1)	32 (25-36)
30 *ad lib.*	3	4.6 (3.9 -5.4)	1.4 (0.93-2.3)	30 (22-36)	9.4 (7.8-10.4)	37 (28-44)

* Range in values.

TABLE III. Summary of Hepatic Storage Data.

Level of protein	No. of animals	Folic acid, γ/g	Biotin, γ/g	Pantothenic acid, γ/g	Riboflavin, γ/g	Nicotinic acid, γ/g
			Ad libitum feeding experiment.			
5	6	0.31 (0.19-0.49)*	0.60 (0.40-0.76)	64 (57- 80)	17 (14-20)	102 (93-115)
7	6	0.34 (0.23-0.48)	0.47 (0.11-0.91)	69 (41- 95)	16 (12-20)	106 (88-119)
10	8	0.58 (0.23-1.4)	0.56 (0.20-1.2)	97 (74-111)	21 (16-29)	127 (108-150)
18	8	0.65 (0.32-0.99)	0.74 (0.36-1.3)	122 (74-164)	30 (24-34)	182 (154-230)
18 + SST	3	0.15 (0.10-0.21)	0.46 (0.31-0.61)	66 (58- 74)	29 (29-29)	143 (139-147)
			Controlled feeding experiment.			
5	7	0.35 (0.13-0.67)	0.60 (0.40-0.88)	58 (38- 78)	20 (16-26)	109 (82-127)
7	6	0.55 (0.35-1.26)	0.89 (0.73-1.22)	88 (69-121)	28 (20-44)	134 (97-205)
10	8	0.48 (0.19-1.55)	0.93 (0.46-1.61)	94 (67-120)	32 (20-43)	156 (133-179)
18	7	1.23 (0.26-2.34)	1.22 (0.45-1.82)	125 (90-152)	48 (35-59)	204 (184-215)
18 + SST	2	0.15 (0.14-0.16)	0.68 (0.56-0.81)	61 (53- 69)	36 (31-41)	182 (175-189)
			High protein feeding experiment.			
18 *ad lib.*	4	0.20 (0.11-0.32)	0.65 (0.57-0.78)	115 (98-141)	31 (24-38)	175 (139-193)
30 controlled	4	0.43 (0.36-0.55)	0.70 (0.64-0.80)	139 (120-151)	32 (31-35)	154 (148-164)
30 *ad lib.*	4	0.34 (0.19-0.44)	0.52 (0.44-0.56)	130 (105-167)	28 (24-31)	144 (137-154)

* Range in values.

ciencies of these factors.[9] Thus a folic acid deficiency was induced indirectly by a reduction in the level of dietary protein. In the *ad libitum* experiment, increasing the level of dietary protein increased the average hepatic storage of folic acid to levels previously established as borderline with respect to a folic acid deficiency.[9] In the second experiment hepatic stores of folic acid were encountered on the higher levels of casein in about half of the animals that were such that a folic acid deficiency should not have been expected.[9] The fecal elimination data demonstrated that these stores of folic acid probably did not come about from increased synthesis of folic acid in the intestine. It is reasonable to conclude that on higher levels of protein intake the folic acid available from bacterial synthesis in the intestine was utilized more efficiently or retained to a greater extent by the animals. It is suggested that a deficiency of certain amino acids or of total nitrogen was responsible for the leucopenia and agranulocytosis that was observed in certain of the animals with the higher hepatic stores of folic acid, although this is not established definitely by the data presented. The recent experiments of Kornberg demonstrating the importance of certain amino acids in blood cell production substantiate this hypothesis.[15]

Although not all animals encountered in the 5% casein or the 18% casein plus SST diets were found to have hepatic stores of pantothenic acid characteristic of a deficiency in this factor,[9] the values found were generally low and emphasize the importance of an adequate protein intake for the maintenance of a normal tissue concentration of this factor. The high renal clearance of administered calcium pantothenate and the normal occurrence of this factor in a combined state in blood and other tissues[16] suggest that in conditions of protein deprivation a substrate for pantothenic acid combination is not made available. The observations made here with respect to protein level

---

[15] Kornberg, A., *Fed. Proc.*, 1946, **5**, 142.

[16] Wright, L. D., Beyer, K. H., Skeggs, H. R., Russo, H. F., and Patch, E. A., *Am. J. Physiol.*, 1946, **145**, 633.

TABLE IV.
Summary of White Cell Data.

Level of protein, %	Duration of feeding, days	Total count, thous. per mm³	Neutrophils, thous. per mm³
		Ad libitum feeding experiment.	
5	11	13.6 (7.8-23.9)* (8)†	2.3 (1.2 -5.8) (8)
5	28	12.5 (8.3-21.3) (8)	1.6 (0.5 -8.9) (8)
5	43	13.3 (5.9-26.7) (7)	1.3 (0.4 -2.4) (7)
5	56	11.5 (6.9-21.3) (7)	1.2 (0.2 -2.5) (7)
5	70	7.7 (4.4-12.3) (7)	0.6 (0.2 -1.1) (7)
5	76	4.8 (3.0- 6.0) (3)	0.3 (0.2 -0.5) (3)
5	82	5.0 (2.6- 6.6) (3)	0.6 (0.2 -0.9) (3)
7	11	17.2 (13.3-21.8) (8)	2.3 (0.7 -4.3) (8)
7	28	14.2 (9.8-22.5) (8)	1.4 (0.4 -3.5) (8)
7	43	13.4 (8.0-18.2) (8)	1.5 (0.3 -4.3) (8)
7	56	10.5 (6.3-15.9) (7)	1.4 (0.2 -3.1) (7)
7	70	10.3 (6.1-18.8) (7)	1.0 (0.2 -2.7) (7)
7	76	6.5 (3.7-11.8) (4)	1.0 (0.1 -2.9) (4)
7	82	6.7 (5.6- 7.7) (2)	0.8 (0.2 -1.4) (2)
10	11	18.0 (13.5-27.8) (8)	2.3 (1.3 -3.1) (8)
10	28	16.8 (9.7-23.7) (8)	1.8 (0.7 -2.9) (8)
10	43	16.9 (12.9-25.2) (7)	2.1 (1.3 -3.2) (7)
10	56	15.9 (12.5-20.3) (8)	2.4 (1.5 -3.4) (8)
10	70	13.0 (7.2-20.1) (8)	1.7 (0.3 -2.9) (8)
10	76	8.7 (4.9-11.6) (4)	1.1 (0.2 -2.1) (4)
10	82	8.3 (7.1-11.3) (4)	0.7 (0.3 -1.3) (4)
18	11	16.2 (8.6-20.9) (7)	2.2 (1.4 -2.8) (7)
18	28	19.7 (14.1-29.5) (8)	1.9 (0.8 -3.2) (8)
18	43	16.2 (13.4-20.9) (8)	1.8 (0.7 -2.3) (8)
18	56	18.9 (14.6-25.5) (8)	2.1 (1.3 -2.9) (8)
18	70	18.1 (16.0-27.8) (8)	2.7 (1.1 -3.8) (8)
18	76	12.0 (10.6-13.1) (3)	1.5 (1.2 -1.9) (3)
18	82	9.7 (7.8-12.7) (5)	1.2 (0.7 -2.0) (5)
18 + SST	11	11.1 (7.3-15.2) (8)	1.1 (0.4 -2.3) (8)
18 + SST	28	11.5 (6.5-17.1) (8)	1.4 (0.1 -2.7) (8)
18 + SST	43	8.4 (5.7-11.0) (7)	0.8 (0.2 -1.8) (7)
18 + SST	56	5.9 (1.7-10.8) (7)	0.4 (0.02-0.7) (7)
18 + SST	70	6.5 (4.2- 8.6) (3)	0.8 (0.3 -1.7) (3)
18 + SST	76	3.7 (3.4- 4.1) (2)	0.2 (0.2 -0.2) (2)
		Controlled feeding experiment.	
5	31	14.3 (6.9-22.2) (8)	1.3 (0.5 -3.0) (8)
5	56	7.4 (3.6- 9.6) (4)	0.5 (0.3 -0.9) (4)
5	67	4.9 (3.4- 6.1) (3)	0.4 (0.03-0.6) (3)
7	31	16.6 (11.5-25.5) (8)	1.4 (0.9 -1.7) (8)
7	56	6.6 (5.9- 7.8) (3)	0.7 (0.6 -0.9) (3)
7	67	5.8 (3.6- 7.7) (3)	0.4 (0.3 -0.6) (3)
10	31	17.0 (12.1-23.4) (8)	1.8 (0.3 -5.3) (8)
10	56	5.3 (4.5- 6.5) (4)	0.7 (0.3 -1.1) (4)
10	67	4.4 (2.7- 6.2) (4)	0.2 (0.04-0.4) (4)
18	31	13.6 (4.7-19.7) (8)	1.0 (0.5 -1.6) (8)
18	56	4.6 (4.0- 5.6) (3)	0.4 (0.4 -0.5) (3)
18	67	5.4 (3.9- 8.0) (4)	0.4 (0.03-0.8) (4)
18 + SST	31	6.2 (4.4- 7.3) (7)	0.5 (0.04-0.9) (7)
18 + SST	56	2.4 (1.9- 2.9) (2)	0.1 (0.1 -0.2) (2)
		High protein feeding experiment.	
18 *ad lib.*	35	7.3 (5.3- 8.4) (4)	0.7 (0.4 -1.0) (4)
30 controlled	35	7.2 (4.3-11.1) (4)	0.5 (0.2 -1.0) (4)
30 *ad lib.*	35	9.5 (6.7-13.1) (4)	0.9 (0.7 -1.3) (4)

* Range in values.
† No. of determinations.

TABLE V.
Summary of Red Cell Data.

Level of protein %	No. of animals	Hematocrit, % avg	Hemoglobin, g/100 ml avg	RBC, million/mm3 avg
		Ad libitum feeding exper.		
5	6	42	14.2	8.6
7	6	45	16.4	9.3
10	8	45	16.4	9.0
18	8	47	18.0	8.9
18 + SST	2	43	15.2	7.6
		Controlled feeding exper.		
5	7	36	12.9	7.4
7	6	43	15.5	8.6
10	8	44	15.9	8.3
18	7	47	17.5	8.8
18 + SST	2	41	16.4	6.7
		High protein feeding exper.		
18 *ad lib.*	4	44	15.9	9.5
30 controlled	4	46	16.5	8.4
30 *ad lib.*	4	45	17.3	8.7

and tissue storage of pantothenic acid confirm similar observations made earlier[3] and offer a possible explanation for the increased resistance of rats receiving high protein diets to induced pantothenic acid deficiency as recently observed by Nelson and Evans.[4]

The low hepatic stores of riboflavin observed in rats receiving diets low in protein confirm the findings of Sarett and Perlzweig[1] and of Czaczkes and Guggenheim.[2] In the controlled feeding experiment the ingestion of the 18% casein diet in amounts resulting in severe restriction of growth was accompanied by hepatic stores of riboflavin in excess of that encountered in stock rats.[17]

The white cell data have been summarized in detail (Table IV) for the purpose of demonstrating the relative rate at which leucopenia and agranulocytosis were induced on the various rations. From the first experiment it may be seen that ingestion of the 5% casein diet induced low total and neutrophil counts at approximately the same rate as did ingestion of the SST-containing diet. As might have been expected from the fecal and hepatic assays, when diets of progressively increasing protein content were ingested, the development of leucopenia and agranulocytosis correspondingly was retarded, and at the conclusion of the experiment was less severe. In the second experiment leucopenia and agranulocytosis developed at approximately the same rate in all the groups except for the SST-fed rats where a combination of food restriction and sulfonamide ingestion resulted in a severe and rapid development of the abnormal blood condition.

The red cell data (Table V) demonstrate that the feeding of low protein or the SST-containing diet was accompanied by occasional low hematocrits, low hemoglobin values, and low red blood cell counts. In a deficiency of folic acid in the rat impairment of white cell formation rather than of red cell or hemoglobin production is the most characteristic blood dyscrasia observed.[18]

Summary. Experiments involving the feeding to rats of diets varying in the level of casein were carried out. The following facts were established:

1. In *ad libitum* feeding the fecal elimination of the B vitamins studied closely paralleled the level of dietary protein. In equalized feeding the fecal elimination of biotin, pantothenic acid, and nicotinic acid was directly proportional to the protein content of the diet.

2. The hepatic storage of the B vitamins studied was correlated directly with the pro-

---

[17] Wright, L. D., McMahan, J. R., Cheldelin, V. H., Taylor, A., Snell, E. E., and Williams, R. J., *The University of Texas Publication*, 1941, No. 4137, 38.

[18] Kornberg, A., Tabor, H., and Sebrell, W. H., *Am. J. Physiol.*, 1944, **142**, 604.

tein content of the diet. This relationship existed both in *ad libitum* and controlled feeding experiments. The hepatic stores of folic acid, biotin, pantothenic acid, and riboflavin found in rats fed the lower levels of protein were indicative of either frank or incipient deficiencies.

3. Leucopenia and agranulocytosis were induced readily by diets low in protein. Under these conditions the existence of the dyscrasia was correlated with the reduced fecal elimination and hepatic storage of folic acid. When diets more nearly optimal with respect to casein level, but in greatly restricted amounts, were fed, leucopenia and agranulocytosis were encountered despite the existence in many of the animals of adequate stores of folic acid. Reasons were presented for attributing the condition to the ingestion of insufficient protein and, more specifically, of certain essential amino acids.

4. Low hematocrits, hemoglobin values, and red cell counts were observed occasionally in rats ingesting low protein diets or *highly purified* diets containing succinylsulfathiazole (SST).

15591

pH Stability of St. Louis Encephalitis Virus.*

Carl E. Duffy. (Introduced by W. M. Stanley.)

From the Virus Laboratories of the Children's Fund of Michigan and the Department of Bacteriology, Wayne University College of Medicine, Detroit, Mich.

Studies on several viruses have indicated that for each virus there is a range of hydrogen ion concentration over which the virus is stable and that at higher or lower pH values the virus activity is destroyed.[1-6] The observation[6] that Japanese B encephalitis virus is most stable at a hydrogen ion concentration near pH 8.5, while the other viruses so far studied were more stable in a less alkaline medium, suggested that the effect of hydrogen ion concentration on St. Louis encephalitis virus (S.L.E.) be studied to determine whether the pH at which the latter virus is stable is similar to that reported for the virus of Japanese B encephalitis. The purpose of the present communication is to report the experiments conducted to determine the hydrogen ion concentration at which St. Louis encephalitis virus is most stable.

The virus used was the Webster No. 3 strain of St. Louis encephalitis virus.† The suspension of virus used in the study was prepared as follows: Swiss mice (3-4 weeks old) were injected intracerebrally with 0.03 cc of a 10% saline suspension of mouse-brain virus. On the 3rd day following inoculation the mice showed signs characteristic for mice infected with S.L.E. The brains of 5 mice were removed and ground in a mortar with sand and sufficient saline to make a 10% suspension of infected mouse brains. Following centrifugation at 1,500 r.p.m. for 5 minutes, 10-fold serial dilutions of the supernatant fluid were made in saline containing 10% rabbit serum. The virus suspension was then titrated intracerebrally in

---

* The writer wishes to acknowledge the technical assistance of Norma H. Feeley during this investigation.

[1] Stanley, W. M., *Physiol. Rev.*, 1939, **19**, 524.

[2] Beard, J. W., and Wyckoff, R. W. G., *J. Biol. Chem.*, 1938, **123**, 461.

[3] Beard, J. W., Finkelstein, H., and Wyckoff, R. W. G., *J. Immunol.*, 1938, **35**, 415.

[4] Finkelstein, H., Marx, W., Beard, D., and Beard, J. W., *J. Infect. Dis.*, 1940, **66**, 117.

[5] Miller, G. L., *J. Exp. Med.*, 1944, **80**, 507.

[6] Duffy, Carl E., and Stanley, W. M., *J. Exp. Med.*, 1945, **82**, 385.

† Kindly supplied by Dr. A. B. Sabin.

TABLE I.
Effect of Hydrogen Ion Concentration on St. Louis Encephalitis Virus.

One cc portions of a 10% saline suspension of virus (intracerebral LD_{50} titer of $10^{-7.8}$) were added to 9 cc portions of a buffer at pH indicated below. Mixtures held at 4°C.

pH of buffer	pH of mixture		Material titrated	Dilutions tested*								LD_{50} titer neg. log. of dilution
	At 24 hrs	At 7 days		10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	
7.1	7.1	7.1	Mixture after 24 hrs	—	5/5†	5/5	5/5	5/5	3/5	1/5	—	7.3
			” ” 7 days	0/5	0/5	0/5	0/5	0/5	0/5	0/5	—	<2
8.2	7.9	7.9	” ” 24 hrs	—	—	—	5/5	5/5	4/5	1/5	0/4	7.5
			” ” 7 days	5/5	5/5	0/5	0/5	0/5	0/5	0/5	0/5	3.5
9.2	8.7	8.4	” ” 24 hrs	—	—	—	5/5	5/5	4/5	0/5	0/5	7.4
			” ” 7 days	6/6	5/5	5/5	1/5	0/5	0/5	0/5	0/5	4.6
10.1	9.5	8.8	” ” 24 hrs	—	5/5	5/5	5/5	5/5	5/5	2/5	0/5	7.8
			” ” 7 days	5/5	5/5	5/5	3/5	0/5	0/5	0/5	0/5	5.2
11.2	10.8	9.7	” ” 24 hrs	3/4	1/5	1/5	0/5	—	—	—	—	2.6
			” ” 7 days	0/5	0/5	—	—	—	—	—	—	<2

* Dilutions were made in saline containing 10% rabbit serum which possessed sufficient buffering capacity to bring all preparations to about pH 7.5.
† The denominator denotes the number of mice tested and the numerator denotes the number of deaths.

3- to 4-week-old Swiss mice. The LD_{50} titer of the virus suspension was $1 \times 10^{-7.8}$.[7]

Buffers of desired hydrogen ion concentration were prepared by adding either 10% sodium hydroxide or 3 N hydrochloric acid to a solution 0.05 M with respect to glycine and to disodium phosphate. The buffers were then autoclaved for 15 minutes at 15 pounds.

Immediately after preparation 1 cc of the 10% saline suspension of virus was added to 9 cc of buffer of the desired pH (Table I) and the mixture was immediately stored at 4°C. The various virus-buffer mixtures were titrated intracerebrally in 3- to 5-week-old mice after 24 hours at 4°C and again after 7 days. The results appear in Table I. It will be seen that the virus is very unstable at or near pH 11 since very little virus was present after 24 hours. After 7 days no virus could be detected in the buffer solution at pH 7.1 nor in the buffer solution which had an original pH of 11.2. From the data presented it would appear that the virus is most stable at pH 8.8. No further titrations of the buffer-virus mixtures were done but 0.03 cc of the mixtures were inoculated intracerebrally into mice after 2 and 3 weeks at 4°C. After 2 weeks virus was still present in the tubes which had pH values, after 7 days at 4°C, of 7.9, 8.4 and 8.8 but was not detected in the tubes having a greater or lesser hydrogen ion concentration. After 3 weeks at 4°C virus was still present in the tubes which had a pH of 8.4 and 8.8, after 7 days at 4°C, but not in the other tubes.

Summary and Conclusions. From the data presented here it would appear that the hydrogen ion concentration at which St. Louis encephalitis virus is most stable is similar to that reported for the virus of Japanese B encephalitis. The former was found, by titration in mice, to be most stable at a pH value near 8.8. The virus of St. Louis encephalitis could still be detected after 3 weeks at pH 8.4 and pH 8.8 but not at higher or lower pH values.

---

[7] Reed, L. J., and Muench, H., *Am. J. Hyg.*, 1938, **27**, 493.

15592

Effect of Activity, Rest, and Natural Sleep upon Blood Pressure of Renal Hypertensive Dogs.

Charles E. Kernodle, Jr., Henry C. Hill, and Keith S. Grimson.

From the Department of Surgery, Duke University School of Medicine, Durham, N.C.

An experimental technic for measuring mean systolic blood pressure and pulse rate of dogs during activity, rest, and natural sleep has been described.[1] This technic has been applied to normal dogs and to dogs with neurogenic hypertension produced by excision of the 2 carotid sinuses, section of one vagus sympathetic depressor nerve trunk, and division of the remaining depressor nerve.[2] Variations of blood pressure were noted in both the control and the neurogenic hypertensive dogs. Blood pressures were highest during activity and standing or feeding and lowest during rest and natural sleep. The reduction of pressure of animals with neurogenic hypertension during rest or natural sleep did not reach levels comparable with those reached by normal dogs.

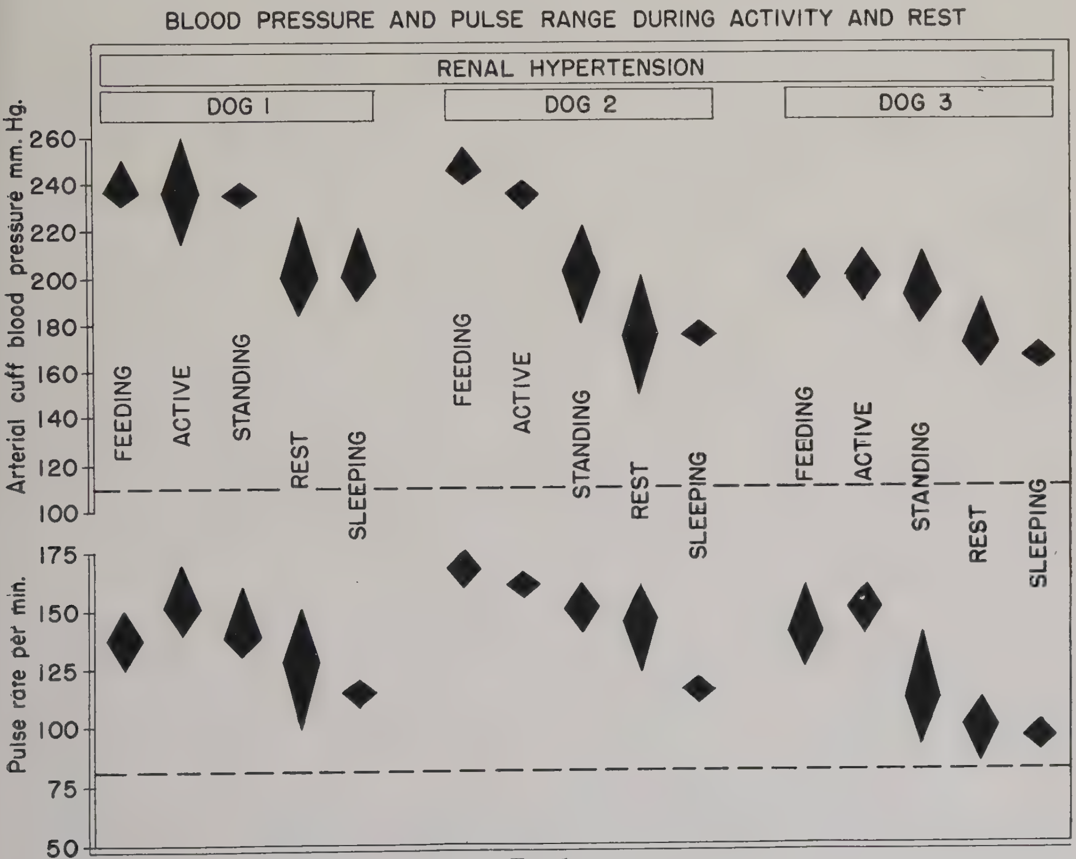


Fig. 1.

Blood pressure and pulse range of 3 renal hypertension dogs. A variable degree of reduction of blood pressure and pulse rate was observed during rest and natural sleep.

---

[1] Kernodle, C. E., Jr., Hill, H. C., and Grimson, K. S., Proc. Soc. Exp. Biol. and Med., 1944, **55**, 64.

[2] Grimson, K. S., Kernodle, C. E., Jr., and Hill, H. C., *J. A. M. A.*, 1944, **126**, 218.

The present experiment was undertaken to determine the effect of activity and rest upon dogs with renal hypertension produced by placing capsules about the kidney, Page.[3] Hypertension was produced in 3 dogs with normal blood pressure by placing silk capsules about both kidneys in 2 (dogs 1 and 2, Fig. 1) and plastic capsules in one (dog 3, Fig. 1). It was produced in a 4th dog by placing a silk capsule about one kidney and removing the other. Blood pressures as determined by direct arterial puncture rose to levels between 190 and 206 mm Hg during the 25 to 36 days that elapsed before the final experiment. Cuffs were then placed about the right iliac and femoral arteries and the animals were prepared for blood pressure determination according to the technic previously described.[1]

After complete recovery from anesthesia blood pressures and pulse rates were recorded at frequent intervals for periods from 49 to 76 hours. The variation and the mean of readings of 3 dogs taken when they were feeding, moving about the cage, standing quietly, lying and resting, or sleeping are illustrated in Fig. 1. The 4th dog with one kidney removed and a silk capsule about the other exhibited unusually high pressures throughout the experiment. Base lines and cuffs were carefully checked without determining a cause. The pressure by direct arterial puncture was 200 mm Hg 9 days earlier. This animal had pressures during the experiment ranging from 275 to 305 while feeding, 265 to 315 while active, 250 to 315 while standing, 270 to 295 while resting, and from 240 to 285 while sleeping. The variations of pulse rate of all 4 dogs during activity and rest roughly paralleled the changes of blood pressure.

Under the conditions of this experiment changes of blood pressure and pulse rate in dogs with renal hypertension are quite similar to those previously reported for dogs with neurogenic hypertension.[2] The reduction of blood pressure and pulse rate that occurs in both types of hypertension does not approach the reduction of levels that occurs in normal dogs.[2]

---

[3] Page, I. H., *Science*, 1939, **89**, 273.

15593

Creatine Concentration and Weight Loss in Denervated Skeletal Muscle.

JOHN HUNTER* AND WILLIAM RUSSELL RAULSTON. (Introduced by L. A. Crandall, Jr.)
From the Division of Physiology, College of Medicine, University of Tennessee, Memphis.

Denervated skeletal muscle undergoes a number of degenerative changes of which atrophy is of especial interest. Hines, Thomson and Lazere[1] have pointed out that muscle strength, weight and creatine content decrease following denervation, subsequently increasing with regeneration of the nerve fibers. Since creatine, in the form of phosphocreatine, plays a vital role in muscular contraction, the present work was undertaken to compare creatine loss and weight loss in denervated skeletal muscle.

Methods. Albino rats were used. The gastrocnemius muscle of one leg of each rat was denervated by removing a section of the sciatic nerve. The opposite leg was left intact and served as a control. Two animals were sacrificed at 2-day intervals after denervation. The intact and denervated muscles were carefully removed, placed directly into tared airtight flasks, weighed and analyzed for creatine.

Of the several methods for determining creatine concentration which have been re-

---

* Physiology Laboratory, Univ. of Michigan, Ann Arbor.

[1] Hines, H. M., Thomson, J. D., and Lazere, B., *Am. J. Physiol.*, 1942, **137**, 527.

TABLE I.

Days after denervation	Body wt	Muscle weight			Creatine concentration					Total creatine			
	g Body wt	g Intact	g Denervated	Denervated / Intact × 100	mg% Intact	mg% Denervated	mg% Denervated avg	mg% (Avg intact conc.) — (Den. conc.)	Den. creatine conc. / Intact creatine conc. × 100	mg Intact	mg Denervated	mg Net creatine loss	Total creatine of denervated / Total creatine of intact × 100
4	135	0.916	0.774	84.5	411	388	373	29	94.4	3.76	3.00	0.76	79.8
4	129	0.794	0.669	84.4	390	367			91.5	2.83	2.39	0.44	84.5
6	240	1.393	1.134	81.5	393	326	338	64	83.0	5.47	3.70	1.77	67.6
6	270	1.734	1.431	82.4	394	349			88.6	6.83	4.99	1.84	73.1
8	162	1.144	0.795	69.5	421	297	312	90	70.5	4.82	2.36	2.46	49.0
8	118	0.856	0.583	68.2	394	326			82.7	3.37	1.90	1.47	56.4
10	224	1.588	1.046	66.0	402	261	298	104	64.9	6.38	2.73	3.65	42.8
10	251	1.870	1.308	69.9	411	334			81.3	7.69	4.37	2.32	56.8
12	251	1.398	0.821	58.8	406	274	292	110	67.5	5.68	2.25	3.43	39.6
12	290	1.949	1.172	60.3	395	310			78.5	7.70	3.63	4.07	47.1
14	291	1.819	0.982	54.1	404	259	246	156	62.4	7.35	2.47	4.88	33.6
14	245	1.624	0.902	55.5	397	239			60.2	6.45	2.16	4.29	33.5

ported[2-4] most investigators have employed the method of Rose, Helmer and Chanutin.[2] For rat muscles we found this method to be satisfactory. In order to simplify the manipulations the following modifications were introduced: Instead of using an autoclave for digestion of the samples, a 4-quart pressure cooker was used. This would accommodate 8 flasks, could be brought to the required pressure in 3 minutes and could be cooled in 3 minutes. Pressure was readily controlled using a laboratory burner. Approximately 1½ hours could be saved by substitution of the pressure cooker for the autoclave in each determination. Alkaline picrate solution was added to the clear protein-free filtrate and the unknown creatine concentrations were compared with standard solutions using a photoelectric colorimeter (Cenco photelometer).

Results. Our results are tabulated in Table I. Inasmuch as dry weight and creatine determination could not be made conveniently on the same muscle, the wet weights were used for comparison. In a second series of rats it was found that average dry weight ratios of denervated and intact muscle are not significantly different from average wet weight ratios on the 14th day after denervation. In this series the mean wet denervated/intact ratio was 0.585 ± 0.045 while the corresponding dry ratio was 0.552 ± 0.057. The standard error of the difference between the 2 means was ±0.02.

Body weights of the animals varied widely among themselves and consequently the actual weight differences in intact and denervated muscles were not directly comparable. The percentage ratio of denervated to intact muscle weights is tabulated. This ratio can be used as an index of the relative weight loss of the denervated muscle. In the present instance this procedure is valid since the intact limb was unaffected by any treatment, as discussed in a previous paper.[5]

The creatine concentration of the intact muscles was found to lie between 390 and 425 mg % with an average value of 401.5 mg %. The 35 mg % variation is beyond the experimental error of the method and is due largely to the variation between animals. Denervation of the muscle of one limb caused little or no progressive effect on the creatine concentration of the opposite intact muscle. For comparison with the creatine concentration of the denervated muscle the average creatine concentration of the intact muscles is probably a better value than any one individual determination since the intact values were relatively constant. The time course of the difference between either the average or individual creatine concentration of the intact muscles and the individual creatine concentration of the denervated muscles parallels the denervated muscle weight loss. The fact that small denervated muscle weight values are associated with small creatine *concentrations* is evident from a correlation coefficient of 0.96 which is highly significant with a probability of much less than 0.01. The significance of this finding is in the fact that while one would expect the smaller denervated muscles to have a smaller total creatine content, there is actually a smaller creatine *concentration* in the denervated muscles which means that total creatine loss is greater than can be accounted for simply on the basis of muscle weight. This implies that if creatine is intimately related to the contractile mechanism, then the impairment of the contractile mechanism with denervation occurs on a steeper gradient, that is, more rapidly and to a greater extent than is reflected by muscle weight loss.

Conclusions. Average dry weight ratios of denervated and intact muscles are not significantly different from average wet weight ratios on the 14th day after denervation. Creatine concentration parallels weight loss following denervation during the experimental period used. Although one determination appears to be as valid as the other in estimating the extent of atrophy it is evident that there is a greater net loss of creatine than can be explained simply on the basis of weight loss. Both weight loss and creatine loss are progressive with time up to 14 days after denervation.

---

[2] Rose, William C., Helmer, Oscar M., and Chanutin, Alfred, *J. Biol. Chem.*, 1927, **75**, 543.

[3] Horvath, S. M., *J. Cell. and Comp. Physiol.*, 1941, **17**, 315.

[4] Seecof, David P., Linegar, Charles R., and Myers, Victor C., *Arch. Int. Med.*, 1934, **53**, 574.

[5] Solandt, D. Y., DeLury, D. B., and Hunter, John, *Am. J. Physiol.*, 1943, **140**, 247.

15594

Potassium vs. Biotin in the Treatment of Progressive Paralysis in Dogs.

Susan Gower Smith. (Introduced by David T. Smith.)

From the Department of Medicine, Duke University School of Medicine, Durham, N.C.

In earlier reports from this laboratory[1,2] a progressive paralysis was described in dogs subsisting on a diet lacking some members of the B complex and very low in potassium, 0.006%. This paralysis was invariably fatal if untreated and ran its course in 12-24 hours after the progressive stage was established. The mineral lack was suspected as a contributing cause, but since the first dog treated with potassium chloride, 700 mg total dose (55 mg/kg), failed to improve and later responded to 1 mg total dose (80 μg/kg) of synthetic biotin, we then centered our attention on the significance of biotin.

While this work was in progress, Elvehjem and his colleagues[3] succeeded in curing with much larger doses of potassium several dogs that had become paralyzed while subsisting on the same deficient diet. Consequently, the next 3 dogs to become paralyzed in this laboratory were treated orally with potassium chloride in much larger doses, 2-5 g total dose (200-500 mg/kg) as compared with the 0.7 g (55 mg/kg) used in the first unsuccessful treatment. This dose level proved entirely adequate, resulting in prompt and complete reversal of the paralytic process.

There is a marked difference in the therapeutic effects of potassium and biotin on the paralysis even in the same animal (Fig. 1). In making therapeutic tests, only animals that had reached the progressive stage of the paralysis were used. In our experience, animals with neck symptoms only are subject to spontaneous recovery, and, therefore, are not reliable for such tests. The paralyzed animal is usually somewhat emaciated, markedly dehydrated, anuric and suffers from complete anorexia during the attack. After treatment with either biotin or potassium chloride and about the time there is evidence of clinical improvement, copious urination occurs.

In the potassium-treated dogs the reversal of the paralysis was very rapid after it was once initiated. There was usually a delay of 3-6 hours before any clinical improvement occurred in our potassium-treated dogs with a dosing schedule of 1 g of potassium chloride in water solution given orally at 30-60 minute intervals. It was rather dramatic to see a dog sprawled on the floor, apparently functionless in neck and all 4 extremities, suddenly stagger to his feet and in a matter of a few minutes regain function to the extent of being able to walk in an almost normal manner. Without further treatment there is a prompt return of appetite, usually some gain in weight and return to normal function while still subsisting on the deficient diet. A much smaller total dose of potassium is required to overcome anorexia than to overcome the paralysis (Fig. 1). A single adequate potassium treatment affords protection from paralysis for 6-10 weeks. The smallest dose curative of paralysis was 200 mg of potassium chloride per kg of body weight. One hundred mg per kg failed to cure, and intermediate amounts have not yet been tried.

In contrast to this, the biotin-treated dogs

---

[1] Smith, S. G., *Science*, 1944, **100**, 389.

[2] Smith, S. G., *Am. J. Physiol.*, 1945, **144**, 175.

[3] Elvehjem, C. A., personal communication, December, 1945.

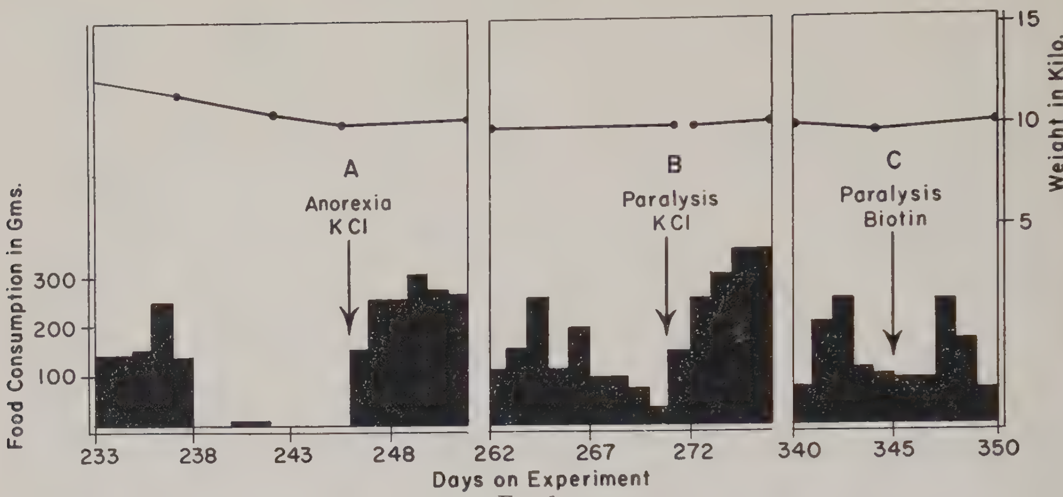


FIG. 1.

Showing the response of Dog No. 168 (weight, 10 kilo) when treated in consecutive attacks of paralysis with KCl and biotin respectively. A. After 9 days of almost total anorexia but no paralysis, 100 mg KCl total dose (10 mg/kilo) given subcutaneously resulted in prompt return of appetite. B. The first attack of paralysis treated with 5 g KCl total dose (500 mg/kilo) given orally, 1 g every ½ hour, resulted in complete reversal of the process 3 hours after treatment was initiated. C. The second attack of paralysis treated with a total dose of 2.4 mg biotin (240 μg/kilo) subcutaneously, 400 γ every hour, resulted in gradual reversal of process over a period of 13 hours. The animal was then maintained with biotin, 800 mg per day.

responded very gradually. There was usually a lag of 8 hours before improvement started even though the material was given parenterally, and during this time the symptoms actually became worse. There was then a gradual return of function over a period of 8-10 additional hours. The appetite returned from the state of complete anorexia during the attack to the low level of intake observed during the period preceding the attack. If no further treatment is given, the dog quickly relapses in from 4 to 48 hours, depending apparently on the vitality and general condition of the dog at the time of the attack.

During an attack of paralysis the serum potassium is definitely lowered. In 2 dogs with paralysis it was found to be 3.5 m eq per liter as compared with 5.36 in a control dog. The sodium, on the other hand, is slightly elevated, being 176.8 in one paralyzed animal as compared with 167.9 m eq per liter in the control.[4] Ferrebee *et al.*[5] have shown that in cases of familial periodic paralysis, a possible human analogue of this syndrome, the serum potassium is lowered during an attack and returns to normal after recovery.

Since the potassium therapy has proved so consistently effective in the treatment of this condition, we wanted to know whether there was any possible contamination with potassium of the biotin solutions used in the treatments reported earlier.[1,2] Fortunately, it was possible to assay the same solutions that were used in these treatments. They were found to be essentially negative although the reagent grade sodium chloride, employed for making the physiological saline (0.85%) used as solvent, according to analysis contained 0.01% potassium. On the basis of this, the saline solutions contained 0.85 μg potassium per cc. The largest total volume used was 50 cc, containing 42.5 μg of potassium. This amount obviously is insignificant as shown by the fact that solutions of biotin containing 4.3 μg of potassium were quite as effective in reversing the paralytic process as this one with 10 times the potassium content. More recently we have obtained positive results with biotin dissolved in distilled water and have failed to produce cures with as much as a gram (100 mg/kg) of potassium chloride given orally.

---

[4] Smith, S. G., reported at meeting of Fed. Biol. Soc., Atlantic City, March 11-15, 1946.

[5] Ferrebee, J. W., Gerity, M. K., Atchley, D. W., and Loeb, R. F., *Arch. Neurol. and Psychiat.*, 1940, **44**, 830.

The Wisconsin investigators failed to get any protection against the paralysis by feeding biotin at a level of 7 μg per kg.[6] We found 50 μg per day completely inadequate to maintain a dog weighing approximately 7 kg.[2] When this amount was doubled, the dog was maintained satisfactorily for 2 weeks.

We have continued to get satisfactory therapeutic responses with biotin as far as the reversal of the neuropathological process is concerned. There is, however, little or no effect on the general condition of the dog, whereas the potassium not only reverses the process in a very short time but has a marked effect on the appetite and general condition. Potassium will maintain these dogs for a long time, possibly indefinitely. One dog has been maintained for 50 weeks and looks entirely normal.

It would appear then that potassium is the primary deficiency and that biotin works indirectly, possibly in mobilizing potassium temporarily or having a synergistic effect when the potassium reserves are too low to operate alone; or, as Ruegamer *et al.* suggests,[6] potassium may increase the intestinal synthesis of biotin. A relationship between biotin and potassium is an interesting possibility. Data are being collected to determine this.

---

[6] Ruegamer, W. R., Elvehjem, C. A., and Hart, E. B., Proc. Soc. Exp. Biol. and Med., 1946, **61**, 234.

15595

Effect of Ascorbic Acid on Diabetogenic Action of Alloxan.

Stanley Levey and Barbara Suter. (Introduced by Arthur H. Smith.)
From the Wayne County General Hospital, Medical Research Department, Eloise, Mich., and Department of Physiological Chemistry, Wayne University, Detroit.

Since it is known that alloxan will react with many substances, and because preliminary unpublished work in this laboratory suggested that ascorbic acid potentiated the action of alloxan, the effect of ascorbic acid on the diabetogenic action of alloxan was investigated.

Experimental. Hooded rats weighing approximately 250 g were used in this study. These animals were fed Purina Dog Chow during the course of the investigation. The animals were fasted 24 hours before the experiment in order to obtain blood samples.

Ascorbic acid was administered intravenously. It consisted of a solution of the free acid made up in distilled water so that 1 cc of solution contained 100 mg of the acid. Most of the animals which received the ascorbic acid, were given a 50 mg dose, while a few received 100 mg. *d*-Isoascorbic acid,* which was used as a control, was given at the same level as ascorbic acid. In all cases the intravenous injections were made in the tail of the rat. Following the immersion of the tail in warm water to dilate the veins, injection was easily accomplished using a 26-gauge needle.

Alloxan was administered intraperitoneally as a 5% aqueous solution in dosage of 100 mg per kg body weight. Previous trials had shown that this amount of alloxan is just below the level necessary to produce diabetes in the test animals.

Mapharsen (3-amino-4-hydroxyphenyl arsinoxide hydrochloride) was administered intravenously to a few rats at a dose level of 2 mg per rat.

---

* The *d*-isoascorbic acid used in these experiments was supplied, through the courtesy of the Charles Pfizer and Company, Brooklyn, N.Y.

TABLE I.
Effects of Various Substances on Production of Alloxan Diabetes. 100 mg of Alloxan per kg Given Intraperitoneally in All Cases. Other Substances Given Intravenously 1 Minute Before the Alloxan.

Treatment	No. animals	No. diabetic	% diabetic
Alloxan	29	5	17.2
Alloxan and 50 mg ascorbic acid	19	9	47.2
Alloxan and 100 mg ascorbic acid	8	4	50
Alloxan and 50 mg *d*-isoascorbic acid	10	1	10
Alloxan and 2 mg mapharsen	5	0	0
Mixture of alloxan and 50 mg ascorbic acid (intraperitoneally)	5	0	0

The general plan of study was to fast the animals 24 hours, obtain blood samples for sugar analysis; one group was then given ascorbic acid intravenously and at a definite time after the intravenous injection, alloxan was administered. A control group received alloxan without having any preliminary treatment. To a third group mapharsen was given intravenously one minute before the alloxan was administered. Blood sugar estimations were made on samples obtained from the tails of the animals by the micro Folin-Malmros method[1] as adapted for the photoelectric colorimeter. Three days later the animals were again fasted and blood sugar estimations were made. At this time urine samples were obtained and qualitative sugar and acetone determinations were carried out. The criterion of diabetes used in this work was a fasting blood sugar of 140 mg % or greater, after the alloxan treatment, while the control value was 112 mg % or less.

Results. In preliminary unpublished work 50 mg of ascorbic acid were given 10 to 15 minutes before the alloxan. The doses of alloxan ranged from 50 to 150 mg per kg of body weight. Ascorbic acid under these experimental conditions produced no effect, that is, the lower doses of alloxan produced no diabetes while the higher doses produced severe diabetes equally well in the group that received ascorbic acid and the group that did not. In addition, this work has also shown that ascorbic acid alone does not produce hyperglycemia.

In succeeding work, 50 mg of ascorbic acid were given one minute before the alloxan. This level of alloxan produced diabetes only infrequently in the animals when no previous treatment with ascorbic acid had occurred. Table I is a summary of the work. In these studies, alloxan alone produced diabetes in 17.2% of the animals. On the other hand, if the animals had previously received ascorbic acid the incidence of diabetes rose to 47.3%. *d*-Isoascorbic acid was used as a control and when given under conditions equivalent to ascorbic acid, diabetes was produced in only 10% of the animals. Thus, it appears that ascorbic acid under the conditions used potentiated the diabetogenic effect of alloxan. Though a few animals were used in the study it is of interest that when 100 mg of ascorbic acid was administered, the incidence of diabetes produced by alloxan increased. This high dose of ascorbic acid must be administered cautiously or death ensues.

In order to determine whether ascorbic acid forms a complex with alloxan which is more diabetogenic than alloxan alone, a few animals received intraperitoneally a mixture of alloxan and ascorbic acid. The mixture consisted of 50 mg of ascorbic acid plus an amount of alloxan equivalent to 100 mg per kg of body weight. None of the 5 animals tested showed hyperglycemia 3 days after this combined treatment.

Discussion. There appear to be 2 possible explanations for the action of ascorbic acid on diabetogenic action of alloxan. One possibility is that ascorbic acid acts as an antioxidant and as such prevents the destruction of alloxan. The fact that alloxan is resistant to the action of strong oxidizing agents, particularly in an acid media, makes this explanation unlikely. Also Yourga, Esselen and Fellers[2] have reported that *d*-isoascorbic acid may act as an antioxidant even for ascorbic acid, yet *d*-isoascorbic acid does not cause any potentiation of alloxan activity when administered just before the alloxan. Another possible explanation is that ascorbic acid decreases the available SH groups in the blood and thus permits more of the alloxan to reach the pancreas. Lazarow[3] has shown that administration of compounds containing the sulfhydryl group, as cysteine, reduced glutathione, and thioglycolic acid, prevent the diabetogenic action of alloxan. Prunty and Vass[4] have shown that feeding human subjects large doses of ascorbic acid decreases the amount of reduced glutathione in the red blood cells. Sulfhydryl groups are known to reduce alloxan to dialuric acid, a substance which has been reported not to possess diabetogenic action.[5]

---

[1] Folin, O., and Malmros, H., *J. Biol. Chem.*, 1929, **83**, 115.

An attempt was made in one series of animals to bind the SH groups by injecting the animals with mapharsen one minute before they received the alloxan. The animals appeared listless after the injections and almost on the verge of coma. When they moved they dragged their bodies around the cage, rubbing their bellies on the floor of the cage. This effect lasted about 3 hours. In no case did hyperglycemia develop in these animals. The dose of mapharsen given was low, but the MLD_{50} for this substance is only 16 mg per kg of body weight.

It appears most probable from these studies that the potentiation of the diabetogenic action of alloxan by ascorbic acid is due to the oxidation of SH groups in the animal and as such allows more alloxan to reach the pancreas.

Summary. Administration of ascorbic acid to rats which are given low doses of alloxan increases the diabetogenic action of the alloxan. To obtain this effect the alloxan must be given within a minute after the administration of the ascorbic acid. *d*-Isoascorbic acid, on the other hand, does not produce the potentiation of the alloxan diabetes.

---

[2] Yourga, F. J., Esselen, W. B., and Fellers, C. R., *Ford Research*, 1944, **2**, 188.

[3] Lazarow, A., Proc. Soc. Exp. Biol. and Med., 1946, **61**, 441.

[4] Prunty, F. T. G., and Vass, C. C. N., *Biochem. J.*, 1943, **37**, 506.

[5] Goldner, M. G., and Gomori, G., *Endocrinology*, 1944, **35**, 241.

15596 P

Primary Herpetic Vulvovaginitis.

Howard B. Slavin and Elizabeth Gavett. (Introduced by William S. McCann.)

From the Departments of Medicine and Bacteriology and the Medical Clinic of the Strong Memorial and Rochester Municipal Hospitals, University of Rochester School of Medicine and Dentistry, Rochester, N.Y.

The varied pattern of clinical disease that may be produced by the agent of the common fever blister is gradually becoming apparent. There have been identified, due to herpes simplex virus, stomatitis and pharyngitis,[1-3] a varicelliform eruption,[4-7] encephalomyelitis,[8-10] and keratitis.[11,12] In

---

[1] Youmans, J. B., *South. Med. J.*, 1932, **25**, 228.

[2] Long, P. H., *J. Clin. Invest.*, 1933, **12**, 1119.

[3] Dodd, K., Johnston, L. M., and Buddingh, G. J., *J. Pediat.*, 1938, **12**, 95.

[4] King, A. D., *Arch. Dermat. and Syph.*, 1939, **39**, 1035.

[5] Wenner, H. A., *Am. J. Dis. Child.*, 1944, **67**, 247.

[6] Barton, R. L., and Brunsting, L. A., *Arch. Dermat. and Syph.*, 1944, **50**, 99.

[7] Lynch, F. W., Evans, C. A., Bolin, V. S., and Steves, R. J., *Arch. Dermat. and Syph.*, 1945, **51**, 129.

[8] Smith, M. G., Lennette, E. H., and Reames, H. R., *Am. J. Path.*, 1941, **17**, 55.

[9] Armstrong, C., *Pub. Health Rep.*, 1943, **58**, 16.

[10] Zarafonetis, C. J. D., Smadel, J. E., Adams, J. W., and Haymaker, W., *Am. J. Path.*, 1944, **20**, 429.

[11] Grüter, W., *Munchen. med. Wchnschr.*, 1924, **71**, 1058.

[12] Gallardo, E., *Arch. Ophth.*, 1943, **30**, 217.

TABLE I.
Isolation and Passage of Herpes Simplex Virus from 3 Cases of Primary Vulvovaginitis.

Patient	Source of virus	First rabbit passage. Corneal inoculation	Second rabbit passage Corneal inoculation*	No. passages intracerebrally in mice
B.H.	Vagina	Keratoconjunctivitis Death from encephalitis Herpetic inclusion bodies in brain	Numerous herpetic inclusion bodies in corneal epithelium	20
L.H.	,,	,,	,,	20
F.E.	,,	Keratoconjunctivitis No further illness Survived	,,	19
	Pharynx	,,	,,	1

* Each animal killed 30 hr after inoculation and cornea examined for inclusion bodies.

TABLE II.
Development of Antibodies for Homologous and HF Strains of Virus Following Primary Herpetic Vulvovaginitis.

	Neutralizing capacity of patient's serum					
	B.H.		L.H.		F.E.	
Strain of virus	A*	C†	A	C	A	C
Homologous	0	6,500	10	1,000	0	160
HF	0	3,000	ND‡	20	0	250

* A—Acute phase serum.
† C—Convalescent phase serum.
‡ ND—Test not performed.

some instances, sufficient evidence has been reported to permit designation of each of these diseases as primary herpetic infections. The evidence upon which rests the case for their primary nature consists of studies of specific antibodies. The finding that human serum sampled at random contains either no antibodies against herpes virus or a high titer[13] has been interpreted to mean that, once infection occurs, the virus persists indefinitely in the tissues. It follows that isolation of herpes virus from lesions of individuals who possess no antibodies against it but who, during convalescence, develop such antibodies, can be accepted as probable evidence of the primary nature of herpetic infection.

The present report describes 3 cases of vulvovaginitis due to herpes virus, proven by isolation of the virus and demonstration of a rising titer of circulating antibodies. These cases are believed to represent the first description of primary vulvovaginitis due to the virus of herpes simplex.

The clinical picture of the primary disease is distinctive. In each of the 3 patients, it consisted of superficial erosions involving considerable areas of the labia minora, adjacent labia majora, and vaginal mucous membrane. The ulcers were tender and usually covered by greyish-yellow membrane. Some of the lesions apparently were autoinoculated for, in every case, opposing surfaces of skin or mucous membrane showed ulcers that obviously had arisen as a result of contiguity. The patients suffered discomfort for a week or longer. The lesions healed without scarification. To date, there has been recurrence in one patient. The recurrent lesion did not resemble that seen initially, but took the form of a cluster of vesicles upon the skin of the labia minora and majora.

As may be seen in Table I, the strains of virus from 2 patients, B.H. and L.H., produced fatal encephalitis in rabbits on first passage. Membranous pharyngitis and vaginitis developed in patient F.E. within 24 hours. Both strains of virus, isolated on suc-successive days, were not encephalitogenic when inoculated upon the rabbit cornea and were immunologically identical. All the strains were productive of "Type A," intranuclear inclusion bodies within epithelial cells of the rabbit cornea (Table I). Cultures of the vaginal ulcers of each case, including attempts to isolate *Corynebacterium diphtheriae,* were not of significance. Darkground examinations of the scrapings from the lesions and repeated serological tests revealed no evidence of syphilis.

---

[13] Burnet, F. M., and Lush, D., *J. Path. and Bact.*, 1939, **48**, 275.

The neutralizing power of undiluted, acute and convalescent phase serums from each patient was determined against decimal dilutions of both the homologous virus and of the HF strain of herpes virus.[14] Each mixture of serum and virus was tested for neutralization in 3 mice, the test carried out with the acute phase serum serving as a control. A summary of the results is given in Table II. The neutralizing capacity of the serums was computed by subtracting the logarithm of LD_{50} of virus plus patient's serum from the logarithm of LD_{50} of virus plus normal rabbit serum. It is apparent that the results of investigation of the 3 cases fulfill the criteria for identification of a primary infection due to herpes virus, as set forth in the introduction to the present report. The similarity and clinically distinctive nature of the cases support this conclusion. It may also be seen in Table II that there is probably no significant difference in the degree to which convalescent serums of patients B.H. and F.E. neutralized both the homologous and HF viruses. However, as tested against convalescent serum of LH, the LH and HF strains appear to be significantly different. Further evidence bearing upon this dissimilarity is contained in an accompanying report.[15]

That herpes progenitalis is essentially a venereal disease[16-18] has attracted little attention in recent years. The husband of patient L.H. had an evanescent, penile sore a few days before his wife became ill. His serum, obtained during the early stages of his wife's illness and again several months later, displayed, on both occasions, a high titer of antibodies against the LH virus. This finding suggests that the husband's infection was of long standing and that conjugal transmission coincided with its recrudescence.

Summary. Primary, herpetic vulvovaginitis may be a clinically distinctive disease. Isolation of the virus from 3 cases and immunological data establishing the probable primary nature of the infection in each have been presented.

---

[14] Flexner, S., and Amoss, H. L., *J. Exp. Med.*, 1925, **41**, 233.

[15] Slavin, H. B., and Gavett, E., Proc. Soc. Exp. Biol. and Med., 1946, **63**, 346.

[16] Unna, P. G., *J. Venereal and Cut. Dis.*, 1883, **1**, 321.

[17] Hruszek, H., *Dermat. Wchnschr.*, 1937, **105**, 1150.

[18] Sharlit, H., *Arch. Dermat. and Syph.*, 1940, **42**, 933.

15597 P

Antigenic Dissimilarity between Strains of Herpes Simplex Virus.

Howard B. Slavin and Elizabeth Gavett. (Introduced by William S. McCann.)
From the Departments of Medicine and Bacteriology and the Medical Clinic of the Strong Memorial and Rochester Municipal Hospitals, University of Rochester, School of Medicine and Dentistry, Rochester, N.Y.

In the course of experiments with the virus of herpes simplex, several strains were compared by means of neutralization tests. A significant antigenic difference between some of the strains was demonstrated.

The antiserum used in all the neutralization tests included in the present report represented a pool of single bleedings of each of 2 rabbits, previously infected with one of the strains under consideration. These animals, which were inoculated upon the cornea with strain FE, developed typical keratoconjunctivitis. Encephalitis did not ensue. They were bled from the heart 32 days after inoculation and the serums were pooled. The animals were not hyperimmunized because of the possibility of decreasing the specificity of their serums.

Three strains of herpes virus were included in the study. The HF strain originally isolated from a human lip vesicle in 1922,[1] and maintained by brain-to-brain passage in mice was employed in its 213th passage. Strains FE and LH were recently isolated from primary herpetic lesions of the genitalia of 2 adult females.[2] Both of the latter strains were also passed serially by intracerebral inoculation of mice. The 19th intracerebral passage in mice of FE and the 20th of LH were used in the neutralization tests.

Neutralization tests were performed by mixing equal volumes of undiluted immune serum and decimal dilutions of the viruses. All tests, including controls, were done as a single experiment on a single day. Neutralization of each virus was controlled concomitantly by mixing normal rabbit serum (pooled serum of 3 rabbits) with appropriate dilutions of the viruses. After 90 minutes at room temperature and 30 minutes in the refrigerator, the mixtures of serum and virus were tested for neutralization by injecting 0.03 ml quantities intracerebrally in adult mice. The animals were inspected daily and deaths recorded over a period of 17 days.

The results of the neutralization tests compared with their controls are shown in Table I. The choice of dilutions of the viruses was based upon a preliminary "pilot-experiment" that yielded a similar result but in which the neutralizing capacity of the serum for decimal dilutions of the viruses was tested in only 3 mice. The quantity of the viruses neutralized was calculated by subtracting the logarithm of LD_{50} of virus plus antiserum from the logarithm of LD_{50} of virus plus

TABLE I.

Results of Neutralization Tests Comparing 3 Strains of Herpes Virus on the Basis of an Antiserum Against One of the Strains.

Strain of virus	Origin	Control Normal rabbit serum Dilution of virus*				Endpoint control (LD_{50})	Neutralization Anti-FE immune rabbit serum Dilution of virus*				Endpoint neutralization (LD_{50})	Quantity of virus neutralized by immune serum (LD_{50})
		10^3	10^4	10^5	10^6		10^2	10^3	10^4	10^5		
FE	Genital	17/20†	15/19	7/20	‡	$10^{4.5}$	6/20	2/20			$\leqq 10^2$	$\geqq 300$
HF	Labial			20/20	6/20	$10^{5.7}$	18/20	5/19	0/20		$10^{2.6}$	1250
LH	Genital		20/20	80/20	0/20	$10^{4.8}$		17/20	2/20	1/20	$10^{3.5}$	20

* Initial dilution of the virus before mixture with serum.

† Numerator = number of mice that died. Denominator = total number of mice inoculated.

‡ Blank space signifies that no test was done with corresponding dilution of the virus.

---

[1] Flexner, S., and Amoss, H. L., *J. Exp. Med.*, 1925, **41**, 233.

[2] Slavin, H. B., and Gavett, E., PROC. SOC. EXP. BIOL. AND MED., 1946, **63**, 343.

normal serum. Failure to include a mixture containing 10^1 dilution of the FE virus plus antiserum prohibits an exact calculation of the endpoint (LD_{50}) of neutralization for this virus. It is highly likely that more than 300 LD_{50} of the homologous virus, FE, were neutralized by the antiserum. In any case, it is apparent that, in the presence of a monovalent antiserum (prepared against strain FE) the FE and HF strains display an antigenic similarity, whereas, in the presence of the same antiserum, the LH strain differs significantly from both of them.

Strains FE and LH were isolated from primary herpetic infections. Convalescent serum of patient FE neutralized the homologous virus and the HF strain to about an equal degree. On the other hand, convalescent serum of patient LH, although it neutralized the homologous virus, showed decidedly less neutralizing capacity against the HF strain.[2] In other words, immune serum of human origin reveals the same antigenic dissimilarity between strain LH and strain HF as does the rabbit antiserum used in the present experiment. In like manner, human antiserum reveals an antigenic similarity between strains FE and HF. These findings lend weight to a supposition that, as judged by the method of comparison used in the present study, the antigenic constitution of strains FE and LH has not been greatly altered by artificial passage in laboratory animals. It remains to compare the strains by means of antiserums prepared against each of them.

15598

Allergic Sensitization to Penicillin: Experimental Results.

W. C. Chu and Windsor C. Cutting.

From the Department of Pharmacology and Therapeutics, Stanford University Medical School, San Francisco, Calif.

Hypersensitivity reactions to penicillin have occurred frequently in patients treated with this antibiotic and also have been produced in healthy men and animals. Some have been attributed to impurities in the penicillin, and some to penicillin itself, although there is a paucity of reports on the pure crystalline preparation. This paper reports the successful production of allergic sensitization, particularly the Arthus phenomenon in rabbits, by the use of crystalline sodium penicillin G and of commercial calcium penicillin.

Previous reports have described clinical sensitization of several types. The most common have been urticaria, angioneurotic edema, vesicular, bullous or papular eruptions, contact dermatitis, the Arthus type of reaction, and a condition resembling serum sickness.[1-3] Reactions have been most frequent after topical application of the drug,[4-8] but have also occurred after parenteral administration following initial topical use,[9] and after parenteral administration only.[10,11] In some cases the symptoms appeared after the initial exposure, and in others at later intervals. Among the more comprehensive studies have been those of Keefer,[12] who

---

[1] Price, D. E., McNairy, D. J., and White, E. L., *J. Am. Med. Assn.*, 1945, **128**, 183.

[2] Editorial, *J. Allergy*, 1945, **16**, 302.

[3] Gordon, E. J., *J. Am. Med. Assn.*, 1946, **131**, 727.

[4] Vickers, H. R., *Lancet*, 1946, **1**, 307.

[5] Barker, A. N., *Lancet*, 1945, **1**, 177.

[6] Pyle, H. D., and Rattner, H., *J. Am. Med. Assn.*, 1944, **125**, 903.

[7] Binkley, G. W., and Brockmole, A., *Arch. Dermat. and Syph.*, 1944, **50**, 326.

[8] Seymour, H. S., *Arch. Dermat. and Syph.*, 1944, **50**, 328.

[9] Haswell, R. E., and Wilkinson, J. F., *Lancet*, 1946, **1**, 308.

[10] Macey, H. B., and Hays, T. G., *U. S. Nav. Med. Bull.*, 1945, **45**, 1143.

[11] Flinn, L. B., *et al.*, *Delaware State Med. J.*, 1945, **17**, 133.

[12] Keefer, C. S., Marshall, E. K., Lockwood, J. S., and Wood, W. B., *J. Am. Med. Assn.*, 1943, **122**, 1217.

found urticaria 14 times in 500 patients with infectious disease treated with penicillin, and Cormia, Jacobsen and Smith,[13] who reported reactions in 10 out of 200 patients.

Preexisting sensitivity has been studied by several reporters. Rostenberg and Welch[14,15] reported that among 144 normal persons injected intradermally with crystalline penicillin, 8 showed positive reactions, and in some, repeated injections produced an Arthus type of reaction. These authors assumed that persons showing the initial sensitivity had previous contact with penicillium mould in nature. Cohen and Pfaff[16] made patch tests with penicillin ointments on 524 human subjects, of whom 5 showed positive reactions.

In animals, McClosky and Smith[17] produced anaphylactic shock in 4 of 11 guinea pigs previously sensitized with commercial penicillin. With the isolated guinea pig uterus, a delayed response thought to indicate sensitivity was irregularly found in animals similarly sensitized.

The following is a summary of our results in animals and in one patient.

Results in Animals. Tests for Preexisting Sensitivity in Guinea Pigs and Rabbits. Eleven normal guinea pigs and 12 normal rabbits were injected intradermally with 100 u of crystalline penicillin G* in 0.1 cc of physiological salt solution. Two guinea pigs showed indurated wheals of 3 cm diameter and 0.5 cm thickness, without redness, lasting for one day. One rabbit showed a red wheal, 3 cm in diameter and 0.5 cm in height, lasting the same time. Simultaneous control injections of the same saline solution produced no lasting skin reaction. Three guinea pigs were tested symptomatically by intravenous injection of 10,000 u of crystalline penicillin G, and 3 for smooth muscle contractions in perfused isolated lungs (bronchi), and on isolated strips of intestine, bladder and uterus with 1,000 u in 50 cc of Tyrode's solution without demonstrable positive reactions.[18] All these smooth muscles reacted typically to applications of histamine. Thus infrequent urticarial reactions may occur in normal animals but typical general sensitivity could not be demonstrated.

Generalized Sensitization in Guinea Pigs. In an attempt to produce generalized, systemic sensitization, 40 guinea pigs were injected according to groups as follows: (1) 3 were given a single subcutaneous injection of 1000 u of crystalline penicillin G in 1 cc of physiological salt solution; (2) 6 were given 1000 u of crystalline penicillin G in 1 cc physiological salt solution subcutaneously and simultaneously 1 cc of a 1:10 dilution of normal horse serum in physiological salt solution at another site; (3) 6 were given 100 u of crystalline penicillin G in .1 cc of physiological salt solution subcutaneously every day for 5 days, and simultaneously 1 cc of a 1:10 dilution of normal horse serum on each day at another site; (4) 5 were sprayed in the nostrils with 1000 u of the penicillin in 1 cc of physiological salt solution on 5 successive days; (5) 3 had a similar amount of penicillin dropped on the conjunctivae on 5 successive days; (6) 7 had 100 units of the penicillin in 0.5 cc of physiological salt solution applied to scarified abdominal skin on 5 successive days; (7) 6 were given 100 u of penicillin in 0.1 cc of physiological salt solution subcutaneously daily for 5 days; (8) 5 were given a single subcutaneous injection of 500 u of penicillin which had been mixed with 0.5 cc of their own serum and incubated at 37°C for 2 hours; and (9) 4 were given a single subcutaneous injection of 500 u of penicillin which had been incubated at 37°C with 0.5 cc of normal horse serum for 2 hours. After intervals of from 18 to 31 days, tests

---

[13] Cormia, F. E., Jacobsen, L. Y., and Smith, E. L., *Bull. U. S. Army Med. Dept.*, 1945, **4**, 694.

[14] Welch, H., and Rostenberg, A., *J. Am. Med. Assn.*, 1944, **126**, 10.

[15] Rostenberg, A., and Welch, H., *Am. J. Med. Sci.*, 1945, **210**, 158.

[16] Cohen, T. N., and Pfaff, R. O., *Arch. Dermat. and Syph.*, 1945, **51**, 172.

[17] McClosky, W. T., and Smith, M. I., PROC. SOC. EXP. BIOL. AND MED., 1944, **57**, 270.

* Supplied by the Squibb Institute for Medical Research.

[18] Chow, Bacon F., and McKee, Clara, *Science*, 1945, **101**, 67.

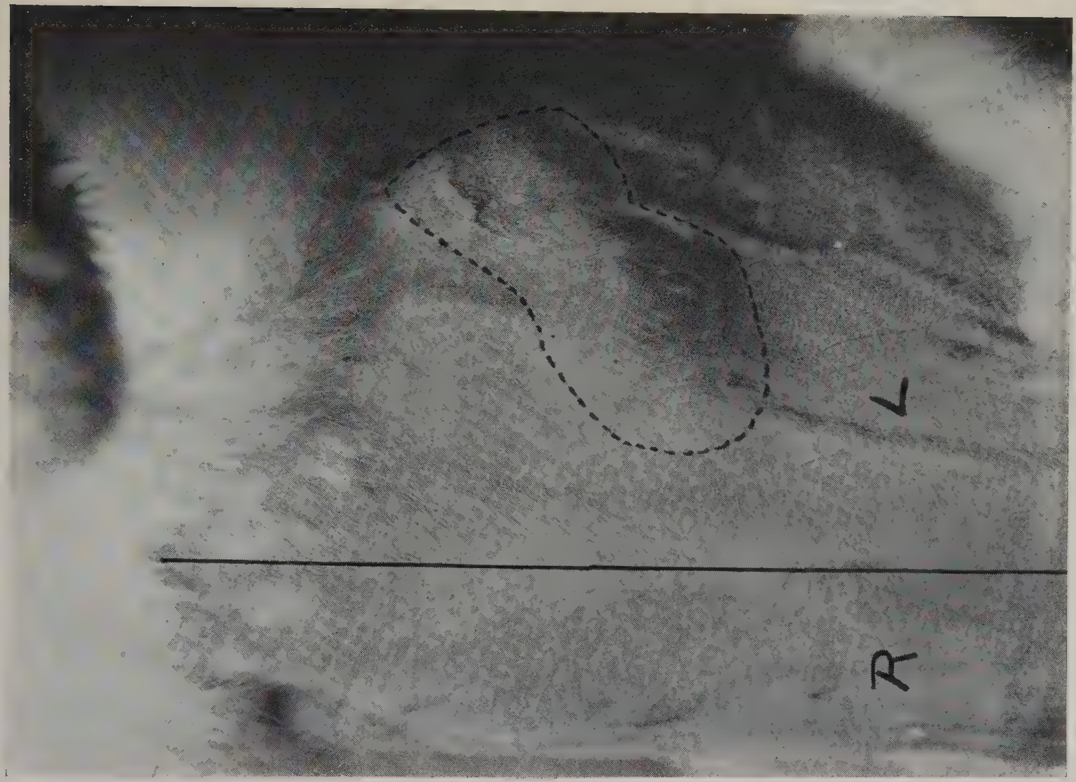


Fig. 1.

Typical Arthus Phenomenon in a Rabbit after Crystalline Sodium Penicillin G.

Shows a hard nodular swelling (broken line) in the left upper abdominal skin (L) after the fifth hypodermic injection, using 10,000 u in 5 cc physiological salt solution, on the thirtieth day after the first injection. Opposite right side (R) shows flat skin after the same injections of saline solution above (control).

for allergic sensitivity were made in a variety of ways.

Using doses of 1000 to 2000 u of crystalline penicillin G in 50 cc of Tyrode's solution, tests were made for smooth muscle contractions in isolated organs† in the following numbers of animals in the first 6 groups: 19, perfused lungs (bronchi); 17, intestinal strip (longitudinal muscle); 13, bladder strip; and 5, uterus strip. None showed a positive reaction, while 5 of the 7 animals simultaneously injected with horse serum showed bronchoconstriction, and one of the 2 tried showed uterine contraction, but none of 5 tested showed contractions of intestine or bladder. However, all of these smooth muscle organs reacted typically to histamine.

Eleven animals from groups 4, 6 and 7 were tested for local sensitivity by the intradermal injection of 1000 u of crystalline penicillin G in 0.1 cc of physiological salt solution, by the intravenous injection of 5,000 u of the penicillin in 0.5 cc of physiological salt solution, and by intravenous injection of 5000 u of the penicillin which had previously been incubated for 2 hours at 37°C with 0.5 cc of the guinea pig's own serum. Also, precipitation tests were made by incubating for 2 hours at 37°C one drop of each animal's serum, obtained before any other tests for sensitivity were performed, with one drop of a solution containing 20 u of penicillin in each cc, and with one drop of this penicillin solution previously incubated with normal guinea pig serum. Only one positive reaction appeared with any of these tests, namely, an urticarial flare with the intradermal injection of penicillin in an animal sensitized by spraying penicillin in the nose. Two animals, additionally tested by Prausnitz-Kuster's method showed no response. For this test, 1 cc of the animal's serum was injected into a normal animal, and again 24 hours later, another 1 cc was injected into the same area.

---

† The methods used for isolated organs throughout were those described in Sollmann, T., and Hanzlik, P. J., *Fundamentals of Experimental Pharmacology*, Second Ed., 1939.

The 9 animals of Groups 8 and 9 were tested for sensitivity by the intradermal injection of 1000 u of crystalline penicillin G, in 0.1 cc of physiological salt solution, the intradermal injection of 0.1 cc of guinea pig or horse serum, and by mixtures of the penicillin solution and the guinea pig serum. Four dermal reactions to penicillin were obtained, 3 with penicillin-guinea pig's serum mixtures and one with penicillin alone. The intravenous injection of 1500 u of penicillin, and lung perfusion with penicillin gave no positive reactions in 3 animals each, although in one animal anaphylactic shock was produced by the intravenous injection of horse serum into the same animal sensitized by the penicillin-horse serum mixture.

Thus it appears that generalized sensitization of the guinea pig by penicillin administered by various routes, with or without incubation with homologous or heterologous serum, and with or without simultaneous sensitization to horse serum, is difficult or impossible to produce. As with preexisting sensitivity, occasional mild reactions may be demonstrated by the intradermal injection of penicillin or mixtures containing it.

Local Sensitization in Rabbits. Local skin sensitization of the Arthus type was studied in rabbits with pure crystalline penicillin G, and with commercial calcium penicillin.‡

Intradermal Injections. Two rabbits were injected 7 times intradermally in the same area of the abdomen at 5-day intervals with 100 u of penicillin G in 0.1 cc physiological salt solution. Both showed slightly red, edematous swellings about 3 cm in diameter and 0.5 cm in height, lasting 24 hours after the 5th injection, and somewhat smaller reactions after the 6th and 7th injections. One animal was simultaneously injected on each occasion on the opposite side of the abdomen with 0.1 cc of a 1:10 dilution of horse serum in physiological salt solution with the appearance of similar swellings following the 3rd to 6th injection, and a less intense swelling on the 7th. Similar repeated injections of physiological salt solution, as controls, produced no local reactions.

Intradermal Followed by Subcutaneous Injections Eight rabbits were injected intradermally twice at intervals of 7 days with 50 to 500 u of penicillin G in 0.1 cc of physiological salt solution. After a lapse of 15 days, injections were continued subcutaneously at 7-day intervals for 4 more injections, using 1000 u of penicillin G in 1 cc of physiological salt solution. Similar control injections with a 1:10 dilution of normal horse serum, or with physiological salt solution, were made on the opposite side of the abdomen. All but one showed dermal swellings at the site of the penicillin injections, similar to, but often larger than, those which received intradermal injections alone. These swellings started with the 1st to 4th injection, were maximal after the 3rd or 4th injection, and diminished thereafter. The serum controls gave similar, but more intense reactions, while the salt solution produced no effect.

Subcutaneous Injections. Nine rabbits were injected subcutaneously at intervals of 3 to 9 days for 5 to 7 injections with crystalline penicillin G, from 2 different lots of the drug. The dose varied from 500 to 10,000 u in volumes of 1 to 5 cc of physiological salt solution. Reactions appeared in 7 rabbits varying from 2 to 12 cm in diameter and from 0.5 to 6 cm in height. The more intense swellings occurred with the larger doses, and were maximal from the 3rd to 5th injection. The swellings were soft and boggy, usually faint to bright red in color, but sometimes without redness. They seldom lasted more than 24 hours, and never became necrotic. Fig. 1 shows a fairly typical reaction in the left upper abdominal skin of a rabbit, chiefly a large wheal, the opposite

---

‡ Supplied by the Squibb Institute for Medical Research.

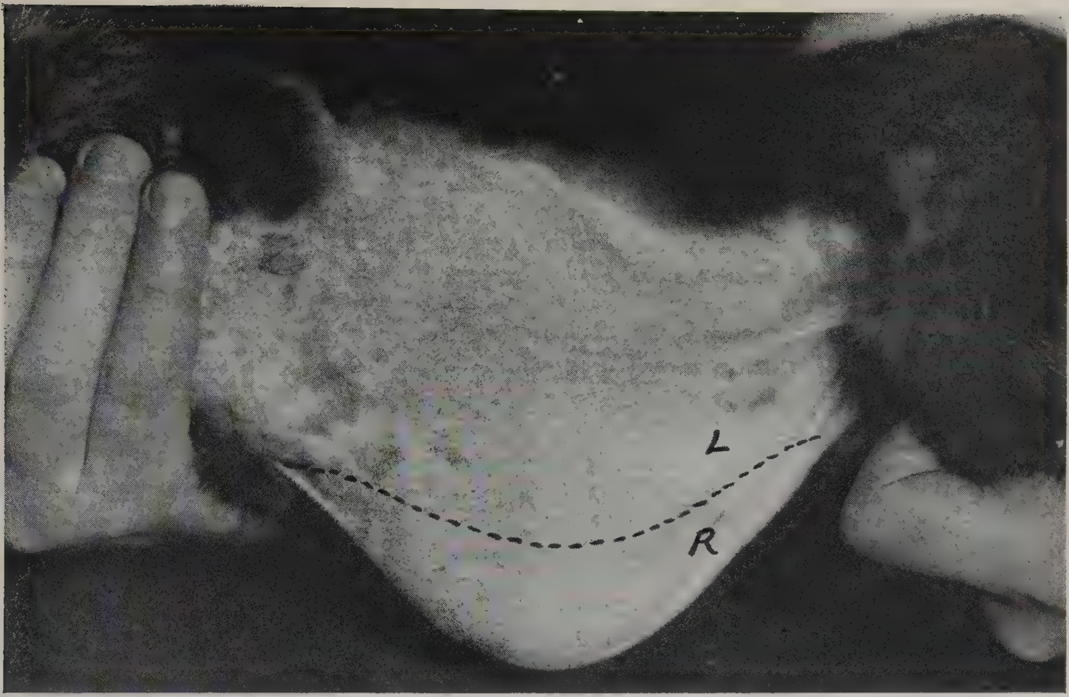


FIG. 2.
Marked Arthus Phenomenon in a Rabbit after Commercial Calcium Penicillin.
Shows a pendant, pouchy swelling in the right abdominal skin (R) after the fifth hypodermic injection of 5000 u in 1 cc of physiological salt solution, and on the twenty-third day after the first injection. The control area injected with saline solution alone in the same way is just above the linea alba (broken line), showing smooth, flat skin (L).

right side of the abdomen showing flat skin (control side).

Subcutaneous Injections with Commercial Calcium Penicillin. Four rabbits were injected subcutaneously with 1000 to 5000 u of commercial calcium penicillin in 1 cc of physiological salt solution at intervals of 4 to 6 days for 5 injections. Two animals were injected similarly on the opposite side with a 1:10 dilution of horse serum and 2 with the salt solution. Moderate to intense reactions to calcium penicillin occurred, similar to those described with pure sodium penicillin, but not more marked. Only one of the 2 control injections with horse serum produced swelling.

Thus, striking reactions like the Arthus type of phenomenon seen after injections of serum could be produced in rabbits by repeated intradermal or subcutaneous injections of either pure or commercial penicillin in saline solution. The route appeared to be unimportant, but larger doses gave greater reactions. Fig. 2 shows one of the more marked reactions to calcium penicillin in the right abdominal skin of a rabbit, the opposite skin above the linea alba being flat and smooth (saline control).

Clinical Case Report. A male physician, aged 40 years, injected himself subcutaneously in one anterior thigh with 300,000 u of commercial penicillin in oil and beeswax, and repeated the injection in the other thigh 2 days later. The injections were superficial and may have been intradermal, in part. Over the next 3 days he took 500,000 u of penicillin by mouth, and after an interval of 4 more days, was given 25,000 u of commercial penicillin intramuscularly in the buttocks every 3 hours for 3 days (25 doses). After the 20th dose the sites of the original penicillin injections in the anterior thigh itched intensely, urticaria appeared, and then tense red swellings. After 5 more injections the lesions were orange-blue, slightly painful to the touch, and looked as though they would become necrotic. The intramuscular injections were then stopped, and the reactions subsided slowly over 2 weeks. A generalized macular eruption also appeared soon after the injections were stopped, most marked over the wrists and ankles, which cleared in 5 days. The local reactions in the thighs appear to have been typical Arthus phenomena, similar to those produced in the rabbits receiving penicillin alone. There is no good reason to believe that oil and beeswax would produce such typically localized reactions.

Discussion. Allergic sensitivity reactions were demonstrated in guinea pigs and rabbits with pure, white, crystalline penicillin G, and with commercial calcium penicillin. These took the form of mild local urticaria after intradermal injections, sometimes without previous known exposure to penicillin, and, more typically, of lesions characteristic of the Arthus phenomenon, some very intense. The reactions with commercial penicillin were no more intense than those with pure penicillin. It is, therefore, apparent that penicillin itself may act, presumably as a haptene in combination with body proteins, as a sensitizing agent, since Chow and McKee have shown that there could be a union between penicillin and serum albumin.[18]

General hypersensitiveness in guinea pigs was not demonstrable with crystalline penicillin, although McClosky and Smith[17] have reported this with less pure penicillin. This discrepancy may depend upon the purity of the preparation, reactivity of the guinea pigs used, or possibly upon differences in technic or dosage. However, it appears that with pure penicillin systemic sensitivity is definitely less important than the local, or contact sensitivity. The skin is an important organ through which penicillin sensitivity is mediated as with some other drugs.

Conclusions. 1. Mild preexisting sensitivity to pure crystalline sodium penicillin G, injected intradermally, was demonstrable occasionally in guinea pigs and rabbits. 2. Typical general sensitivity could not be demonstrated with pure penicillin G applied to guinea pigs by local or parenteral routes. 3. Local sensitization of the Arthus type phenomenon was produced consistently by repeated injections into rabbits of pure sodium penicillin G, or commercial calcium penicillin. These reactions were comparable to those produced by similar injections of horse serum, but somewhat less intense. 4. Similar local sensitization was observed in a patient injected with commercial penicillin in oil and beeswax. 5. Accordingly, the purest obtainable crystalline penicillin can act as a sensitizing agent with manifestations like those of native protein, and the skin is the outstanding organ for the seat of the allergic reaction.

15599

Comparative Numbers of Fungiform and Foliate Papillae on Tongues of Domestic and Wild Norway Rats.*

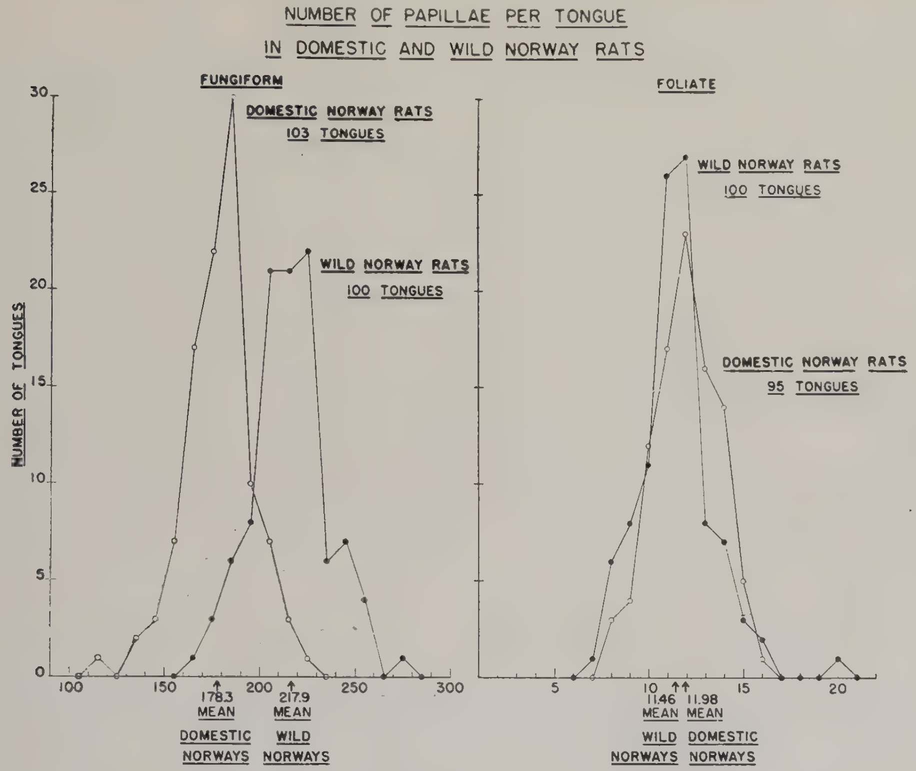
HAROLD S. FISH AND CURT P. RICHTER.

From the Psychobiological Laboratory, Phipps Psychiatric Clinic, The Johns Hopkins Hospital.

Previous physiological and anatomical studies have revealed marked differences between domestic Norway rats which have lived for many years under laboratory conditions and wild Norway rats which have

---

* A grant from the International Health Board of the Rockefeller Foundation aided this investigation. The Rodent Control Office of the City of Baltimore supplied the wild Norway rats; Mr. Jack Spencer of the Fish and Wildlife Service in Gainesville, Fla., supplied most of the Alexandrine rats.



A.

B.

FIG. 1.

just been trapped from the cellars, yards and alleys in a large city. It was found: (1) While wild rats have a high resistance to poisoning with thiourea, as much as 2000 mg per kg body weight being required to kill them, domestic rats show a great sensitivity, succumbing to doses as small as 5 mg per kg body weight.[1] Thus, over a 400-fold difference exists between the 2 strains in the amounts of thiourea needed to kill them. Furthermore, the wild rats killed with thiourea do not show pulmonary edema or pleural effusion, the characteristic symptoms of domestic rats poisoned with thiourea. (2) While wild rats react to sounds of high frequency by furiously attacking the source of the stimulation, a large percentage of domestic rats manifest so-called running fits and convulsions and die in a coma.[2] (3) Owing to a retrogression of their adrenal cortices, domestic rats have much smaller adrenal glands.[3,4]

The present study has revealed that a marked difference exists also between the number and appearance of fungiform papillae on the tongues of these 2 strains of Norway rats.

The gross appearance of the tongues of domestic rats, the number and size of their fungiform and foliate papillae, were described in a previous paper.[5] In the present study a comparison has been made between these previous findings and data obtained with essentially the same technic from recently trapped wild rats. Observations were made also on a few Alexandrine rats *(Rattus rattus alexandrinus)* which, next to the Norways, are the most common and widely distributed rats throughout the world, inhabiting ships and seacoast towns, and building their nests in rafters and upper stories of buildings rather than burrows or cellars. They are less ferocious than wild Norways and prefer an herbivorous rather than an omnivorous diet.

---

[1] Dieke, S. H., and Richter, C. P., *J. Pharmacol.*, 1945, **83**, 195.

[2] Griffiths, W. J., Jr., *Science*, 1944, **99**, 62.

[3] Donaldson, J. C., PROC. SOC. EXP. BIOL. AND MED., 1928, **25**, 300.

[4] Rogers, P. V., and Richter, C. P., in press.

[5] Fish, H. S., Malone, P. D., and Richter, C. P., *Anat. Rec.*, 1944, **89**, 429.

The 103 domestic rats used in the previous study ranged in age from 8 hours to 466 days and came from a colony of rats of the Wistar strain which had been interbred with rats of the hooded strain from the colony of Dr. E. V. McCollum. No new strain has been added during the past 20 years.

The 100 wild Norway and 20 Alexandrine rats used in the present study ranged in weight between 75 and 487 g and constituted a random sample taken from thousands of rats which were trapped from the yards, alleys and cellars in the residential districts of the city of Baltimore. Most of the Norways were killed and injected within a few days after their capture but most of the Alexandrines were used for various experiments beforehand.

The tongues were injected with methylene blue through the carotid arteries by the same technic as was used previously for the laboratory animal.[5] The fungiform and foliate papillae were observed under a dissecting microscope and plotted on large charts for counting. The papillae were counted usually shortly after the removal of the tongues but, in a few instances, they were placed in formalin and were not counted for 10-20 days.

Results. These observations showed that in gross appearance the tongues of wild Norway rats closely resemble those of domestic rats, but their fungiform papillae are smaller and more uniform in size and are more liberally distributed along the sides of the "intermolar eminence," or large hump near the base of the tongue.

Fig. 1A compares the number of fungiform papillae found on the tongues of 103 domestic Norways and of 100 wild Norways. Calculation leads to mean values and standard deviations of 178.3 ± 15.1 papillae per tongue for the domestic rats and 217.9 ± 19.0 for the wild rats. Thus, there is a statistically significant difference between the number of fungiform papillae in the 2 strains of rats, with the mean for domestic Norways falling 17% lower.

The lowest and highest counts for domestic Norways were 114 and 221 fungiform papillae per tongue, with only one rat having a count above the mean for wild Norways. The wild rat counts ranged from 167 to 271, with only 3 tongues having papillae counts below the mean for domestic rats.

The foliate papillae of wild rats tend to have a more uniform size and shape than do those of laboratory animals. In general the individual papillae are shorter and only occasionally deviate from the typical groove-like structure. The "V," "Y" and "H" forms characteristic of laboratory rats are rare. At both ends of the row of papillae the slits tend to become shorter, and anterior ones appear as small pits.

Fig. 1B shows the number of foliate papillae per tongue for 95 domestic and 100 wild Norways. The range for the domestic rats was from 8 to 16 and for the wild Norways from 7 to 20. The respective means and standard deviations are 11.98 ± 1.72 for domestic and 11.46 ± 1.98 for wild rats. Although there was no essential difference in the average number of foliate papillae, the wild rats showed a definitely wider range of variation.

For the 20 Alexandrines the corresponding values are 191.5 ± 23.8 (range 147 to 231) fungiform papillae per tongue, and 13.5 ± 2.4 (range 9 to 18) foliate papillae. The mean number of fungiform papillae in this smaller series of Alexandrine rats thus falls between the means of the domestic and wild Norways, while the mean number of foliate papillae is somewhat greater than that found for either strain of Norway rat. The character and distribution of the fungiform papillae resemble those of the laboratory rat tongue, while the foliate papillae are more like those of the wild Norway.

Discussion. The results of these observations indicate that in some way the domestic rats of our colony have been selectively bred for a lower number of fungiform papillae. This selective breeding may have taken place before the rats arrived in our colony, or it may have taken place during the last 20 years. In this period the only selective breeding that has been practiced wittingly, and that only to a limited extent, has been for running activity in revolving drums.

The reduced number of fungiform papillae may be an instance of the effect of domestication which results when rats no longer have to forage for their food or to depend on finer taste differentiations in the selection of their food. Before such a conclusion could be drawn, it would be necessary to know whether domestic rats from other colonies show a similar reduction in the number of papillae. Whether or not a direct relation exists between keenness of taste and the total number of fungiform and foliate papillae (and, indirectly, the taste buds contained in them) remains unknown. Taste-threshold tests made before and after sectioning of taste nerves to the various papillae may provide an answer to this question.

Finally, attention may be called again to the opportunity offered by these findings for studies in the field of genetics. An actual count of the papillae makes it possible to express a feature of a strain of rats in numerical rather than in qualitative terms.

Summary. 1. The number of fungiform and foliate papillae were counted on the tongues of 100 recently trapped wild Norway rats and of 20 wild Alexandrine rats, and compared with the number on the tongues of 103 domestic Norway rats. 2. The number of fungiform papillae on the tongues of the domestic Norway rats ranged from 114 to 221, with a mean of 178.3, while for the recently trapped wild Norway ancestors it ranged from 167 to 271, with a mean of 217.9, and for the Alexandrine rats it ranged from 147 to 231, with a mean of 191.5. The mean of the domestic Norway rat thus showed a 17% reduction from that of its wild ancestor. 3. The number of foliate papillae ranged from 8 to 16 for the domestic rats, with a mean of 11.98, and ranged from 7 to 20 for the wild Norways, with a mean of 11.46. The 2 strains thus showed essentially the same number of foliate papillae, but both showed fewer than are found in Alexandrine rats, in which the number of papillae ranged from 9 to 18, and had a mean of 13.5. 4. In general, both types of papillae were more uniform in size, structure and distribution in the wild than in the domestic strains.

15600

Effect of Riboflavin Deficiency on Endochondral Ossification of Mice.*

Barnet M. Levy and Ruth Silberberg.

From the Department of Oral Pathology, Washington University School of Dentistry, and the Laboratory of Jewish Hospital, St. Louis, Mo.

The role of riboflavin in the maintenance of normal body growth has been demonstrated in rats, mice, pigs,[1] and monkeys.[2] Growth is retarded if riboflavin is omitted from the diet, and young rats are born with skeletal abnormalities[3] (especially micromelia) if the mothers are fed a riboflavin-deficient diet during pregnancy. These observations indicate a disturbance of endochondral ossification. The present investigation was undertaken in order to determine which phase of this process is dependent upon the presence of riboflavin, and to compare the find-

---

* Aided by the Louis M. Monheimer Memorial Fund.

[1] Eddy, W. H., and Dalldorf, F., *The Avitaminoses. The Chemical, Clinical and Pathological Aspects of the Vitamin Deficiency Diseases*, 3rd Edition, The Williams & Wilkins Company, Baltimore, 1944.

[2] Cooperman, J. M., Waisman, H. A., McCall, K. B., and Elvehjem, C. A., *J. Nutrition*, 1945, **30**, 45.

[3] Warkany, F., and Nelson, R. C., *Arch. Path.*, 1942, **34**, 375.

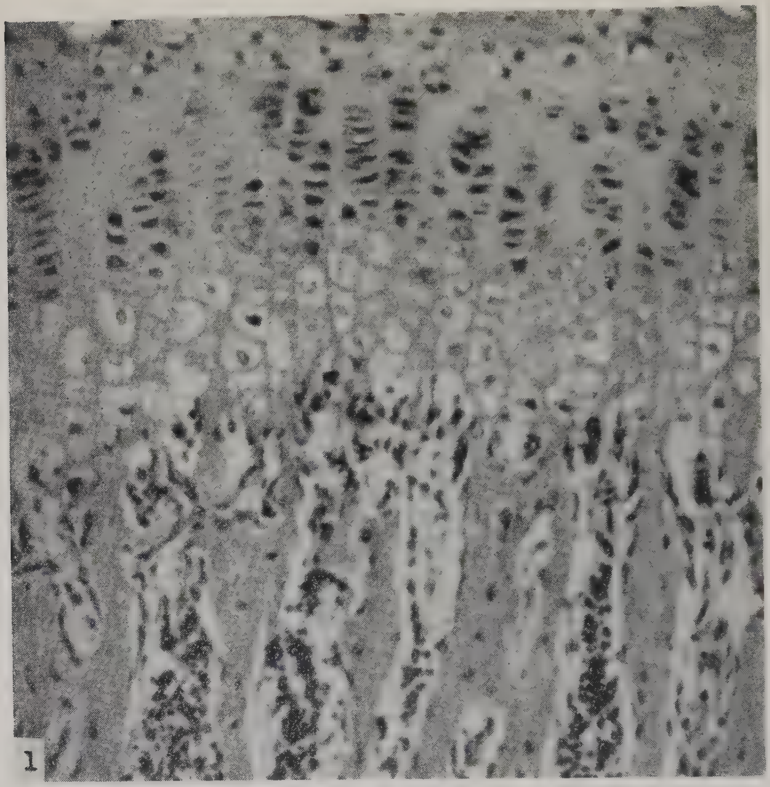


FIG. 1.
Growth zone of the upper tibia of a female mouse of Strain C57, black, 42 days of age. Mag. 200 ×.

ings with those obtained in pantothenic acid deficiency.[4]

Material and Methods. Twenty-four growing and 11 adult male and female mice of Strain C57, black, were fed a diet which was complete except for the lack of riboflavin.†

The ration was composed as follows:

Sucrose	68 %
Vitamin test casein	18 ”
Vegetable oil	10 ”
U.S.P. salt mixture No. 2	4 ”
Cod liver oil	1 ”
Thiamin	0.2 mg%
Nicotinic acid	1.0 ”
Pyridoxine	0.2 ”
Choline chloride	1.0 ”
Inositol	100.0 ”
Calcium pantothenate	10.0 ”

The growing mice were 30 days old, and the adult mice were 9 or 14 months of age at the beginning of the experiments. The animals were kept on the deficient diet for periods of one, 2, 3 or 4 weeks and, in the adult series, for 5 weeks. After each of these periods 2 or 3 animals were killed. In addition, 4 animals fed the deficient diet for 4 weeks were returned to a normal diet of Purina Chow for periods of 6 or 10 days and were then sacrificed. At the autopsy the tibia and femur were removed together, fixed in 10% formalin, decalcified with 45% formic acid, sectioned and stained with hematoxylin and eosin. All mice kept on the deficient diet either failed to gain weight or made slight gains up to 4 g. After one week the hair appeared greasy and stringy. After 2 weeks, there was slight loss of hair especially around the mouth and on the back. As the deficiency progressed, several small ulcers appeared around the mouth,

---

[4] Levy, B., and Silberberg, M., PROC. SOC. EXP. BIOL. AND MED., 1946, **63**, 380.

† We are indebted to Merck and Company for the liberal supply of vitamins used in this study.

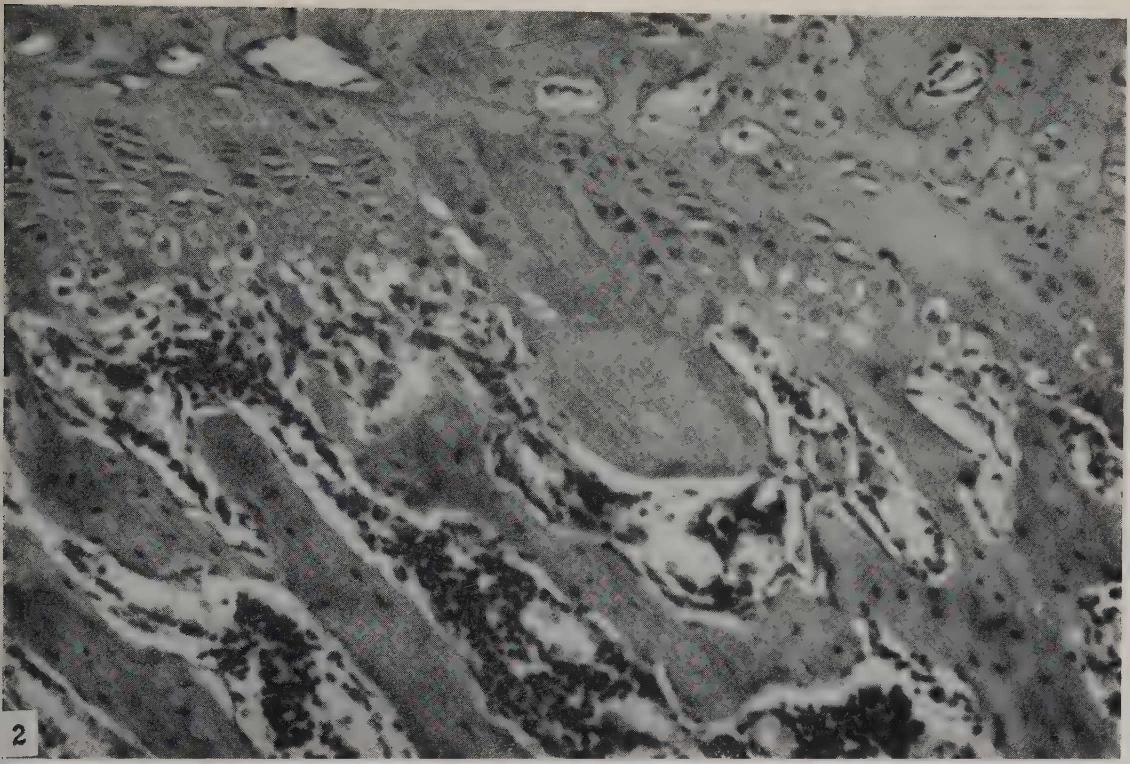


Fig. 2.

Growth zone of the upper tibia of a female mouse of Strain C57, 58 days of age and kept on a riboflavin deficient diet for 28 days. The growth zone is greatly narrowed and traversed by a thick plug of degenerated cartilage. Mag. 200 ×.

and scabs were noted on the back, ears and tail.

Histological Findings. Growing Animals. The epiphyseal zone of the animals kept on the deficient diet for one week was of normal width, containing 10 columnar and 4 hypertrophic cells. There was slight increase in the intercartilaginous matrix. The nuclei of the columnar cells were clumped and more irregular than usual. There was active breakdown of the hypertrophic cartilage. The subepiphyseal spicules were normal in number and thickness. The metaphyseal capillaries were well filled and accompanied by numerous osteoblasts, as is usual. The bony shafts were likewise unchanged.

In animals kept on the deficient diet for 2 weeks, the growth zone was narrower. In the single cartilage cell row, the number of columnar cells was decreased to 6 or 8 and that of the hypertrophic cells to 2 or 3. Both types of cells were smaller than normal, and the columns were irregular. There was hyalinization of the matrix and, here and there, amorphous plugs began to form, traversing the entire width of the growth zone. The spicules were fairly thick, but shorter than ordinarily. The metaphyseal capillaries were less filled than previously and partly collapsed. Osteoblasts were present in moderate numbers, but they were more oblong than usual, and clusters of osteoblasts were found in grooves at the surface of spicules without osseous substance being deposited between them. The shafts showed slightly enlarged vessels.

Feeding of the deficient diet for 3 or 4 weeks caused a marked narrowing of the growth zone (Fig. 1 and 2). There were 6 columnar and one or 2 hypertrophic cells in a single cartilage cell row, both types of cells being smaller than usual and showing irregular nuclei. The matrix was even more hyalinized, and moderately wide amorphous

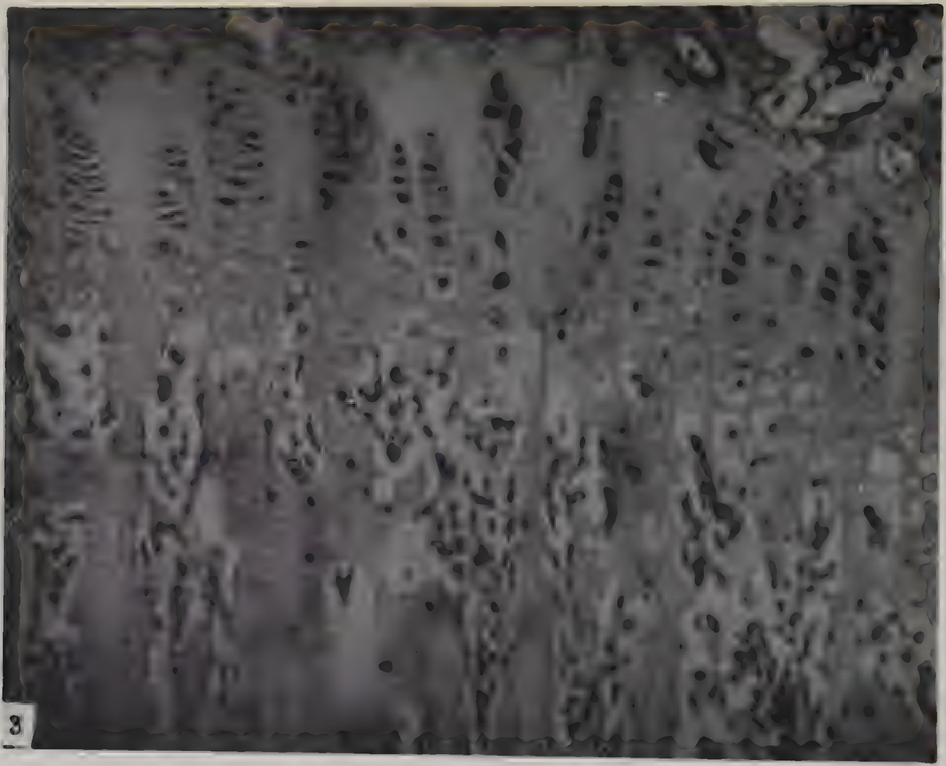


FIG. 3.

Growth zone of the upper tibia of a female mouse of Strain C57, black, 68 days old, kept on a riboflavin deficient diet for 4 weeks and refed an adequate stock diet for 6 days. The growth zone is wider than in Fig. 2. A plug of degenerated cartilage is seen in the middle of the field, being pushed toward the metaphysis. Mag. 200 ×.

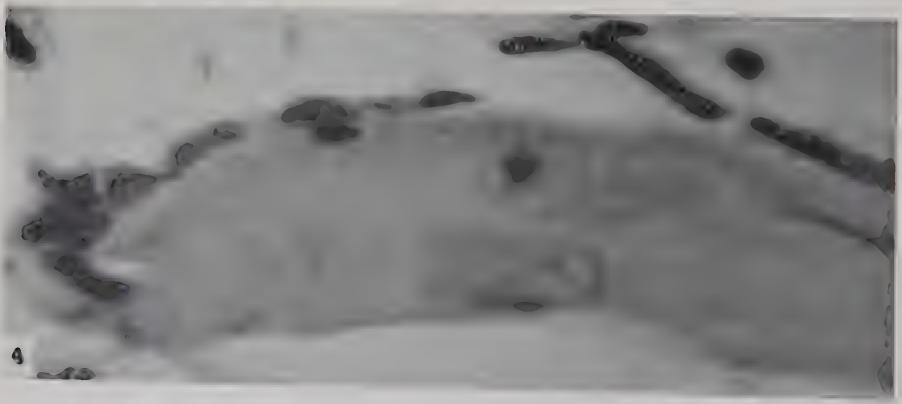


FIG. 4.

Section through the metaphysis of the same mouse as in Fig. 2. A bony spicule surrounded by a single layer of elongated spindle-shaped osteoblasts. Mag. 1275

plugs of destroyed cartilage appeared between the preserved cartilage rows. Still, the metaphyseal capillaries were breaking down the hypertrophic cartilage and in some places had advanced almost to the border of the columnar cartilage, where bone was being deposited. The trabeculae were further shortened. However, there was no trans-

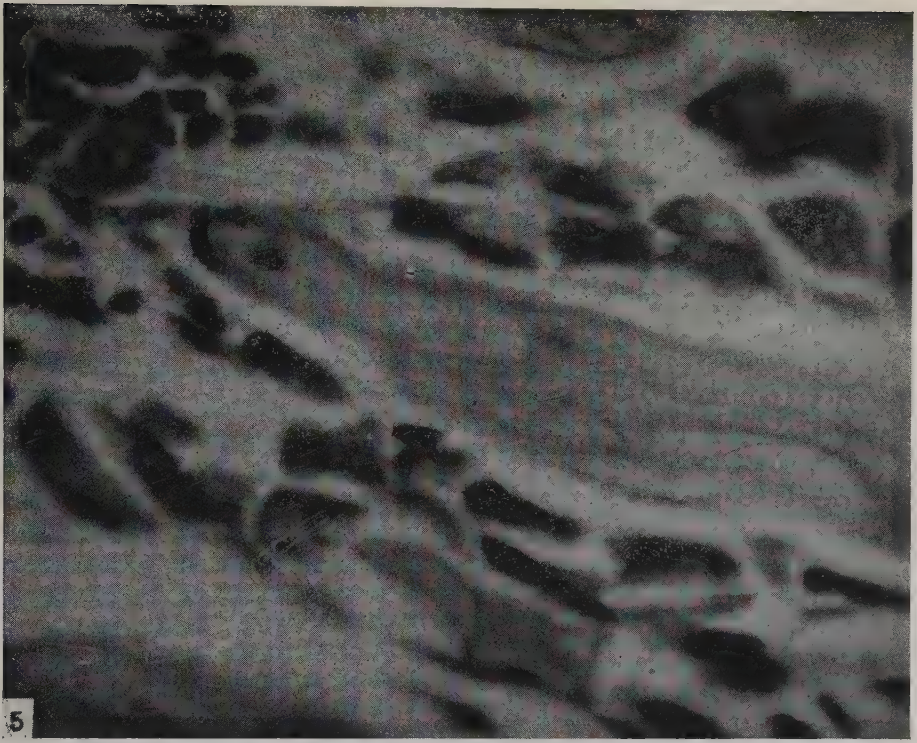


FIG. 5.
Section through the metaphysis of the same mouse as in Fig. 3, showing a bony spicule surrounded by large osteoblasts. Mag. 1275 ×.

verse bony plate, as is found in conditions associated with arrest of growth. Osteoblasts were scantier than before, and they were spindle-shaped (Fig. 4). They formed small clusters here and there, suggesting an attempt to form spicules. No osseous matrix, however, was deposited between them. The shafts were thin and showed only a scattering of spindle-shaped osteoblasts at the endosteal surface.

The above findings were present in male as well as in female mice. After one or 2 weeks, the males showed somewhat more marked changes than the females, but after 3 or 4 weeks the findings were about equal in both sexes.

Upon refeeding, there was a rapid return of the growing cartilage to normal conditions (Fig. 3). After 6 days the epiphyseal plates were enlarged, due to an increase in size as well as in the number of the cells. There were now 8 to 9 columnar and 2 to 3 hypertrophic cells. There was still increased hyalinization of the matrix, and an occasional small amorphous plug of degenerated cartilage could be found. These plugs, however, did not traverse the entire growth zone, but reached from the level of the most distal hypertrophic cartilage cells to about the middle of the width of the columnar zone. It thus seemed as if repair of the cartilage was accomplished by replacement from the undifferentiated cartilage and that the degenerated tissue was pushed down toward the metaphysis. A few eosinophilic leucocytes were noted in the vicinity of these plugs. The metaphyseal capillaries were well filled and accompanied by numerous large osteoblasts (Fig. 5), some of which were still spindle-shaped, whereas others were polygonal, as is usual. The trabeculae were somewhat longer than in the corresponding animal which was not refed.

After 10 days of refeeding, repair had made further progress. The growth zone was of normal width. There were now 10 to 12 columnar and 3 hypertrophic cells, the columnar cells being somewhat increased over the normal and indicating an energetic resumption of growth. The metaphysis was well vascularized. The spicules were of normal length and width, and they were covered by thick layers of large polygonal osteoblasts. Repair was accomplished more rapidly in females than in males.

Adult Animals. In control animals the epiphyseal disc was composed of a small amount of inert degenerated cartilage, sealed off on both sides by a bony plate. The bony shaft and the epiphyseal spicules were lined by large, ovoid osteoblasts. In the mice kept on the deficient diet for 4 and 5 weeks, no changes were noted in the growth zone. However, the number of osteoblasts was decreased, and the bony structures were covered by a discontinuous layer of spindle-shaped cells.

Summary and Conclusions. In growing mice, riboflavin deficiency decreases both the rate of growth and of ossification, but intensifies the degeneration of the cartilage. These changes represent the histological basis for the inhibition of lengthwise growth seen in riboflavin-deficient animals. As compared with the changes seen in pantothenic acid deficiency, lack of riboflavin inhibits the proliferation of the cartilage to a lesser extent, whereas it produces degeneration of the cartilage, not seen in pantothenic acid deficiency. As in pantothenic acid deficiency, males are more sensitive to the lack of the vitamin than are females.

In adult mice, the endosteal and peritrabecular osteoblasts undergo atrophy.

15601 P

Infection Experiments with the Hookworm (*Bunostomum phlebotomum*) in Calves.

ROY L. MAYHEW.

From the Louisiana Experiment Station, Louisiana State University, Baton Rouge.

The calves used in these experiments were obtained from the L. S. U. Dairy Department as soon after birth as possible, usually within 36 hours, and raised under parasite-free conditions in the animal building of the Veterinary Science Department.

Three animals 2½ to 13 months old have been inoculated with infective larvae by mouth. All had begun passing eggs by between 64 and 83 days after receiving the larvae. One animal developed a diarrhea lasting about 10 days at approximately 30 days after inoculation.

Three other calves were inoculated by placing larvae on the skin of the flank. These animals were then fastened in stanchions in such a manner as to prevent licking the larvae from the skin, in one instance for 42 days, another 67 days, and in the case of the 3rd for 97 days. Two of these calves developed a very severe bloody diarrhea 31 and 38 days after the application of the larvae, which continued for 10 days in one, and for 18 days in the other. A very marked skin irritation developed over the area of application of the larvae in all 3 animals. Two of these animals began passing eggs on the 57th and 65th days after the application of the larvae. The third calf with the most severe diarrhea and which became the most emaciated, remained negative to the end of the experiment, 133 days.

Three additional calves began passing hookworm eggs at between 60 and 115 days of age after being raised under conditions free of parasitic infection. The animal that was positive at 115 days was not examined between the 78th and 115th day and it is believed that a positive examination could have been secured earlier than the 115th day. It seems evident that these 3 animals secured their infective larvae during the few hours between birth and their transfer to the Veterinary Science Department.

Summary. These experiments indicate that infection with the hookworm parasitic in calves takes place by way of the skin as well as orally and that the larval stages are capable of producing severe symptoms of parasitosis. The prepatent period may be as short as the 57th day and may extend to at least 79 days.

15602

Derivatives of Diphenylsulfone, Related Sulfoxides and Sulfides in Experimental Tuberculosis.*

B. L. Freedlander and F. French.

From the Harold Brunn Research Institute, Mt. Zion Hospital, San Francisco, Calif.

Many investigators have reported favorable results in experimental tuberculosis with derivatives of 4,4′diaminodiphenylsulfone, including promin and diasone, and the related sulfone, promizole. In the following experiments several new derivatives of diphenylsulfone, a number of related sulfoxides and other allied compounds were evaluated for their effect in experimental tuberculosis, both *in vitro* and *in vivo*. An effort was made to determine the relationship between chemical structure and chemotherapeutic activity.

Methods. The bacteriostatic studies were initially performed on the avirulent strain of tubercle bacilli, No. 607, using Proskauer and Beck synthetic media, at a pH of 7.2. The chemical compounds were dissolved in propylene glycol to make 0.5% solutions. Subsequent dilutions were made directly into the media. Each tube was inoculated with one loopful of fresh culture of tubercle bacilli. The appearance of a pellicle was taken as the criterion of growth; the cultures were read at the end of 2 and of 4 days. Compounds showing a favorable bacteriostatic action were repeated, using the virulent culture H37Rv. The cultures were read at the end of 3 weeks. As the differences between the bacteriostatic effect on the avirulent and the virulent cultures were insignificant, only the results of the experiments performed with the avirulent culture are reported.

Animal Experiments. Approximately 9 guinea pigs, averaging 350 g weight, were used to evaluate each compound. The animals were inoculated in the left groin with 0.01 mg of H37Rv tubercle bacilli. The drugs were administered either orally or hypodermically. For oral administration, in most cases, the finely ground drug was mixed with ground rabbit pellets; in the other cases the drugs were suspended in tragacanth solution and given with a pipette. The food was supplemented by fresh vegetables. For hypodermic administration the drugs were ground with either cotton seed oil or a 1% beeswax-sesame oil mixture. All experiments were terminated at the end of 6 weeks. Pathology was evaluated numerically by the method of Sweany, Sher, and Kloeck,[1] in which the maximum gross involvements of the organs were given the following numerical ratings: lymph nodes 8, spleen 24, liver 28 and lungs 40. Hemoglobin determinations were made at the end of the experiments.

Results. The *in vitro* bacteriostatic results are recorded in Table I. The com-

---

* The present report is part of a cooperative investigation on tuberculosis, and has been supported by funds provided by the Committee on Medical Research of the National Tuberculosis Association, the California Tuberculosis Association, the San Francisco Tuberculosis Association, the Columbia Foundation of San Francisco, and the Rose Lampert Graff Foundation of Beverly Hills, Calif.

[1] Sweany, H. C., Sher, B. C., and Kloeck, J. M., *Am. Rev. Tuberculosis*, 1946, **53**, 250.

TABLE I.
Tuberculostatic Action.

Compound	Chemical formula	Conc. 1-part-in:
4,4′Diaminodiphenylsulfone	$H_2NC_6H_4SO_2C_6H_4NH_2$	60,000
2,4′ ,,	,,	40,000
2,2′ ,,	,,	10,000
3,3′ ,,	,,	10,000
4-Nitro,4′acetylaminodiphenylsulfone	$O_2NC_6H_4SO_2C_6H_4NH \cdot COCH_3$	10,000
4-Benzylideneamino,4′aminodiphenylsulfone	$C_6H_5 \cdot CH{=}N{-}C_6H_4SO_2C_6H_4NH_2$	10,000
4,4′Diaminodiphenylsulfoxide	$H_2NC_6H_4SOC_6H_4NH_2$	40,000
4,4′Diacetylaminodiphenylsulfoxide	$H_3COCHNC_6H_4SOC_6H_4NHCOCH_3$	10,000
4-Aminodiphenylsulfoxide	$C_6H_5SOC_6H_4NH_2$	100,000
4-Nitro,4′aminodiphenylsulfoxide	$O_2NC_6H_4SOC_6H_4NH_2$	60,000
4-Nitro,4′acetylaminodiphenylsulfoxide	$O_2NC_6H_4SOC_6H_4NHCOCH_3$	10,000
4-Chloro,4′aminodiphenylsulfide	$ClC_6H_4SC_6H_4NH_2$	800,000
4-Iodo,4′aminodiphenylsulfide	$IC_6H_4SC_6H_4NH_2$	800,000
4-Chloro,4′acetylaminodiphenylsulfide	$ClC_6H_4SC_6H_4NHCOCH_3$	400,000
4-Chloro,4′aminodiphenylsulfone	$ClC_6H_4SO_2C_6H_4NH_2$	10,000
4-Iodo,4′aminodiphenylsulfone	$IC_6H_4SO_2C_6H_4NH_2$	10,000
4-Iodo,4′Nitrodiphenylsulfone	$IC_6H_4SO_2C_6H_4NO_2$	10,000

pounds, 4,4′diaminodiphenylsulfone and diasone, were included for purposes of comparison and internal standardization. 2,4′-Diaminodiphenylsulfone was slightly less bacteriostatic than the 4,4′ derivative; 2,2′diaminodiphenylsulfone and the 3,3′ derivative were the least tuberculostatic of the 4 isomers. 4-Nitro,4′acetylaminodiphenylsulfone and 4-benzylideneamino,4′aminodiphenylsulfone showed only minimal bacteriostatic action *in vitro*. 4,4′Diaminodiphenylsulfoxide was slightly less inhibitory than the corresponding sulfone. 4-Nitro,4′aminodiphenylsulfoxide was slightly superior in tuberculostatic action to the 4,4′diaminodiphenylsulfoxide. Acetylation of the above compounds, in general, prevented the inhibitory effect. 4-Aminodiphenylsulfoxide showed a higher bacteriostatic effect than did the corresponding diamino derivative.

The halogenated derivatives of the sulfides inhibited growth in very high dilutions; 4-chloro,4′aminodiphenylsulfide, as well as the corresponding iodo derivative was bacteriostatic in dilutions of 1:800,000. Acetyl derivatives of these sulfides showed high bacteriostatic action. The halogenated sulfones, on the other hand, showed no tuberculostatic action. *p*-Aminobenzoic acid antagonized the bacteriostatic action of the sulfones and sulfoxides, but not that of the halogenated sulfides. All compounds lost most of their inhibitory action in the presence of 20% horse serum.

The *in vivo* results are summarized in Table II. The isomers of diaminodiphenylsulfone showed the following order of decreasing therapeutic effect: 4,4′; 2,4′; 2,2′. This order is the same as the order of the *in vitro* tuberculostatic effects. 4-Nitro,4′acetylaminodiphenylsulfone and 4-benzylideneamino,4′aminodiphenylsulfone were approximately as effective as 4,4′diaminodiphenylsulfone and diasone. 4,4′Diaminodiphenylsulfoxide, given hypodermically, was as effective as the corresponding sulfone. Given orally, however, its therapeutic effect was comparatively slight. 4,4′Diacetylaminodiphenylsulfoxide showed a considerable effect orally but it was much less effective when given subcutaneously. While the spleens of the animals treated with 4,4′diaminodiphenylsulfone or its benzylidene derivative were large and cyanotic, the spleens of the animals treated with the sulfoxides did not show this effect. None of the halogenated compounds were effective *in vivo*. 4-Iodo,4′-aminodiphenylsulfone was considerably less toxic than the corresponding sulfide. Guinea pigs tolerated a daily dose of 75 mg of the sulfone and only 8 mg of the sulfide.

Discussion. It is interesting to note that

TABLE II.
Summary of *in Vivo* Experimental Data.

Group	Compound	Dosage, mg	Avg path. rating	Path. ratio $\frac{\text{controls}}{\text{treated}}$	Avg wt gain	Hglb.
A	4,4′Diaminodiphenylsulfone	50 daily, oral	33.7	1.77	240	11
	4-Nitro,4′acetylaminodiphenylsulfone	100 ” ”	25.8	2.33	213	10.5
	Controls		60		167	11.4
B	2,4′Diaminodiphenylsulfone	50 ” ”	43	1.09	179	12
	2,2′Diaminodiphenylsulfone	50 ” ”	55.2	0.85	141	11
	Controls		47		118	11.4
C	Diasone*	180 ” ”	19.9	2.26	194	9.4
	Controls		44		175	10.1
D	4-Benzylideneamino,4′aminodiphenylsulfone	100 ” ”	18.9	2.53	213	10.4
	Controls		48		223	10.9
E	4,4′Diaminodiphenylsulfoxide	100 ” ”	47	1.28	113	10
	4,4′Diacetylaminodiphenylsulfoxide	100 ” ”	21	2.86	54	9
	4-Aminodiphenylsulfoxide	50 ” ”	53	1.13	74	10.6
	4-Nitro,4′aminodiphenylsulfoxide	100 ” ”	44	1.36	103	11
	4-Nitro,4′acetylaminodiphenylsulfoxide	80 ” ”	53	1.13	96	11
	Controls		60		167	10.9
F	4,4′Diaminodiphenylsulfoxide	130 hypo 3× wk	18	2.17	172	11.5
	Controls		39		259	10.6
G	4,4′Diaminodiphenylsulfoxide	90 ” q. 4 days	22	2.60	191	11.1
	4,4′Diacetylaminodiphenylsulfoxide	90 ” ” ” ”	34	1.70	201	9.7
	Controls		57.3		160	11.1
H	4-Iodo,4′aminodiphenylsulfone	75 daily oral	44	1.09	191	11
	” ” ”	50 hypo, q. 2 days	42.1	1.14	142	11.1
	Controls		48		223	11
	4-Chloro,4′aminodiphenylsulfide	8 daily, oral	36.7	1.13	116	11.2
	” ” ”	8 ” hypo	41.4	1.06	60	11
	4-Chloro,4′acetylaminodiphenylsulfide	20 ” oral	41.2	1.06	101	11.6
	” ” ” ”	8 ” hypo	37	1.18	87	11.8
	4-Iodo,4′aminodiphenylsulfide	10 ” oral	41.6	1.05	115	13
	Controls		43.7		169	12.8

* Diasone obtained through the courtesy of Abbott Laboratories, Chicago, Ill.

in the sulfoxide group therapeutic effects have been found which are as significant as the therapeutic effects of diaminodiphenylsulfone and its derivative, diasone. Diaminodiphenylsulfoxide has been shown, previously, to exhibit a high therapeutic effect in streptococcus and gonococcus infections in mice.[2–4] This compound has also been found to inhibit tumor growth in mice.[5] Buttle *et al.*[2] found that acetylation did not reduce the therapeutic activity of 4,4′diaminodiphenylsulfoxide in animal streptococcus infections to the same degree as did acetylation of the corresponding sulfone. We have found 4,4′-diacetylaminodiphenylsulfoxide given orally to be as effective as the nonacetylated sulfoxide administered hypodermically in tuberculous guinea pigs.

The compound, 4-nitro,4′aminodiphenylsulfoxide, showed only a slight therapeutic effect on oral administration. The acidity of the gastric juice may have decomposed this derivative. In this connection we have observed that this compound is altered on contact with acids or on long standing. Buttle[2] found the benzylidene derivative of 4,4′-diaminodiphenylsulfone to be low in toxicity and to be more effective than the parent compound in pneumococcus infections in mice and monkeys. They reported that mice would tolerate daily repeated doses of approximately 500 mg/kg. In our experiments on tuberculous guinea pigs a favorable therapeutic effect was obtained with doses of 200 mg/kg per day.

---

[2] Buttle, G. A., Dewig, T., Foster, G. E., Gray, W. H., Smith, S., and Stephenson, D., *Bioch. J.*, 1938, **32**, 1101.

[3] Rosenthal, S. M., Bauer, H., and Elvove, E., *U. S. Publ. Health Rep.*, 1939, **54**, 1317.

[4] Girard, A., Ray, A., and Richard, G., *Nature*, 1937, **140**, 283.

[5] Boyland, E., and Mauson, E. H., *Biochem. J.*, 1938, **32**, 1982.

Summary. A series of derivatives of diphenyl sulfone, sulfoxide and sulfide were tested for *in vitro* tuberculostatic activity and for antituberculous therapeutic activity in guinea pigs. The following compounds, in addition to the reference materials 4,4′-diaminodiphenylsulfone and its derivative diasone, showed high *in vivo* activity: 4-benzylideneamino,4′aminodiphenylsulfone; 4-nitro,4′acetylaminodiphenylsulfone; 4,4′diaminodiphenylsulfoxide (hypo); 4,4′diacetylaminodiphenylsulfoxide (oral). 4-Chloro, 4′aminodiphenylsulfide and the corresponding iodo derivative were highly tuberculostatic *in vitro* but were devoid of *in vivo* activity.

15603

Biological Factors Involved in Poisoning Rats with Alpha-Naphthyl Thiourea (ANTU).*

Curt P. Richter.†

From the Psychobiological Laboratory, The Johns Hopkins Medical School.

From modern studies in the fields of nutrition and biochemistry much has been learned regarding the principles involved in the feeding of animals to keep them alive and in good health; but little has been learned regarding the principles involved in feeding animals substances to destroy them. When animals are kept in captivity this latter objective can be readily attained, often unwittingly; when, however, animals are not confined and have full access to all kinds of foods in their natural states it is less readily attained.

The use of poisons for killing animals by feeding involves at least the following considerations: (1) The poisonous compound must be highly toxic to the animals that are to be killed; (2) it must be readily acceptable in lethal amounts; (3) after surviving the ingestion of sublethal amounts of the compound animals must not be able to develop too high or too prolonged a tolerance; (4) the poison must be as free as possible from taste and odors, by means of which animals once having survived poisoning, are able to recognize it on a second exposure and this devolep a "refusal" response.

In the present paper an attempt has been made to place the study of these different qualifications of a poison—toxicity, acceptance, tolerance and refusal—on a controlled experimental basis.

The substance used for this purpose was alpha-naphthyl thiourea (ANTU), a substance which has been shown to be highly toxic for domestic and wild Norway rats, doses of 1-5 mg killing the average-sized rat. On the basis of the results of these and of other similar tests this substance was recommended as a rat poison and is now actually being used as such on a wide scale.[1]

Method. Sixty-four adult hooded and albino male domestic rats, ranging in age from 82 to 228 days and in weight from 227 to 313 g were used for these experi-

---

* Started under a contract recommended by the Committee on Medical Research between the Office of Scientific Research and Development and The Johns Hopkins University and continued under a contract between the Medical Division, Chemical Corps, U. S. Army, and The Johns Hopkins University.

† With the assistance of Mrs. Romaine Randall.

[1] Richter, C. P., *J. A. M. A.*, 1945, **129**, 927.

ments. At these ages Norway rats have essentially the same high sensitivity to ANTU while younger rats are much less sensitive.[2] The rats came from our own colony. They were the offspring of albino Wistar rats which, approximately 20 years ago, were crossed with hooded and tan rats from the colony of Dr. E. V. McCollum.

To determine the acceptability of ANTU, the rats were kept on our stock diet‡ for a 10-day period, and then were divided into 9 groups of 6 to 10 rats each, which were given the stock diet mixed with ANTU in 9 different concentrations, ranging from 0.0005% to 10.0%, as follows:

Group	% concentration ANTU food	No. of rats
I	10	6
II	2	10
III	1	6
IV	0.1	6
V	0.05	6
VI	0.01	6
VII	0.005	6
VIII	0.001	9
IX	0.0005	9

The rats were kept in individual cages 8″ x 10″ x 12½″, containing a nonspillable food cup and an inverted water bottle. Records were taken daily of the food intake and the rats were weighed weekly and at every time a change was made in their diets. The cups were weighed and refilled to the same weight (100 g) each day. During the time that the rats received the plain stock diet, the food cups were weighed and filled at 11 a.m. every day; while on the day when they were to receive the poisoned diet, the cups were weighed as before at 11 a.m. but were returned empty and were not filled with the poisoned food until 4:30 p.m. Thus, the rats remained without food for 5½ hours. During this time of day, however, rats ordinarily eat little or nothing. The object was, first, to expose to the poisoned food, rats which would be as uniformly hungry as possible, and, further, to avoid at the time of this exposure any disturbing laboratory noises which might be present during the earlier part of the day.

The alpha-naphthyl thiourea was used in the form of a fine powder (80-120 micra particle size), which mixes readily with the stock diet. The drug and the diet were mixed for 15 minutes in a kitchen electric mixer to distribute the poison evenly in the food. For the lower concentrations the powder was thoroughly mixed with a small amount of food in a mortar before it was placed in the mixer with the full amount of food.

To determine the tolerance and refusal which develop when the rats have survived a sublethal dose of ANTU, the surviving rats were continued on their various concentrations of ANTU food for a period of 14 days; then they were given ANTU-poisoned food in a concentration (2%) which consistently killed 100% of all previously unpoisoned rats. The rats which survived the first day of this poisoning continued to receive the 2% ANTU food for a total of 5 days. Then they were returned to the unpoisoned stock diet for a period of 16 days, after which they were exposed for one day to both the unpoisoned stock diet and that containing 2% ANTU mixture. The survivors of this test were returned for a 13-day period to the unpoisoned stock diet and then were tested again for one day with 2% ANTU mixture.

The tolerance was measured in terms of the number of toxic units the animals ingested with impunity. Each "toxic unit" was taken to be 5 mg per kg body weight, or the amount of ANTU which, when administered by stomach tube, very consistently kills all rats from our colony.

Results. Acceptance Tests. All of the rats in Groups I, II, III, and IV, which received the 4 highest concentrations of ANTU food (10, 2, 1, and 0.1%) died within the first 24 hours after the poisoned food was placed in the food cups. All of the rats from the other 5 groups survived. Fig. 1A gives a typical record of one of the rats which had access to a 2% concentration of ANTU food. The bottom chart shows the intake

---

[2] Dieke, S. H., and Richter, C. P., PROC. SOC. EXP. BIOL. AND MED., 1946, **62**, 22.

‡ Graham flour 72.5%, casein 10.0%, butter 5.0%, skim milk powder 10.0%, calcium carbonate 1.5%, sodium chloride 1.0%.

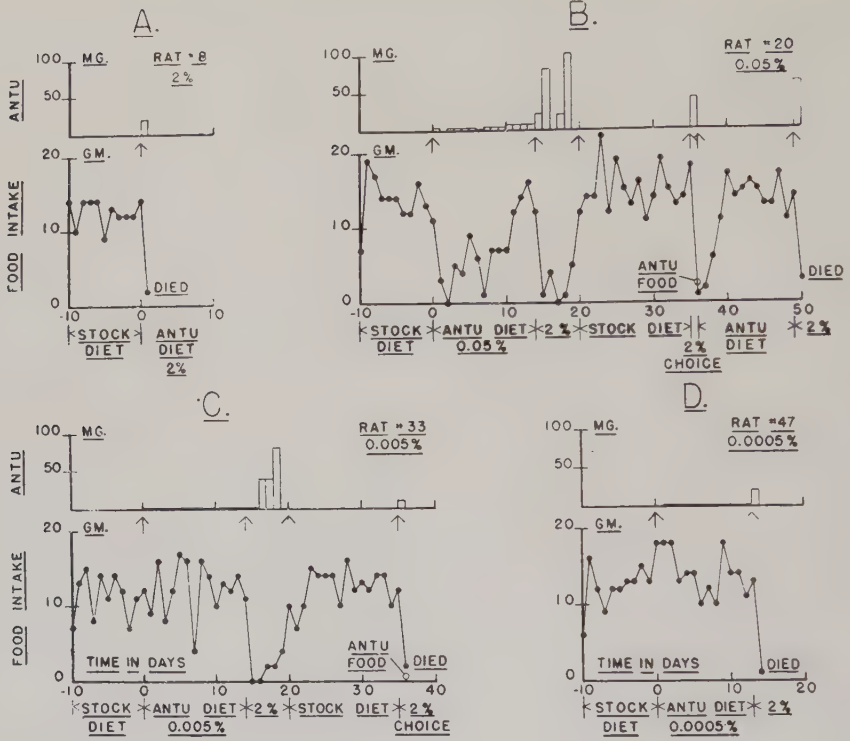


FIG. 1.

Typical individual food and ANTU intake records for rats from 4 different groups which received 2%, .05%, .005%, and .0005% concentrations of ANTU food respectively. The ordinates give the intake of food in grams and of ANTU in milligrams; and the abscissæ give the time in days.

of the stock diet for the 10-day preliminary period and of the ANTU-poisoned food for the first day. The top chart shows the intake of ANTU in milligrams. The food intake dropped from a daily average of 13 g for the 10-day period on the stock diet to 2 g for the first day on the poisoned food.

Table I summarizes the results, giving for each group the average age and body weight; the average daily intake of unpoisoned food for the 10-day preliminary period; the average intake of poisoned food for the first day. For the 10-day period the intake of stock diet ranged from 12-15 g for the different groups of rats. On the first day of the poisoning the rats of the first 6 groups ate much less of the food than they had previously eaten of the unpoisoned food. With these amounts the rats of the first 4 groups ingested lethal doses (from 1.4 to 62 toxic units on the average). The rats of the 5 other groups ingested an average of 1.2 toxic units or less and survived.

Evidence at hand indicates that when exposed to the higher concentrations of ANTU-poisoned food, these previously unpoisoned rats ate less, not because they disliked the food, but because the effects of the poisoning appeared before they could eat their usual amounts. Observations made on many rats while they were eating the poisoned food showed that when first exposed they ate the poisoned food freely in all concentrations as high as 20%. They gave no evidence of refusing to eat it freely, until suddenly, usually after less than 10 minutes, they stopped eating, the ingested poison apparently having made them sick. On unpoisoned stock diet rats ate for from 30 to 60 minutes and ingested 4 to 5 g, definitely more than was eaten by the rats on the poisoned diets.

TABLE I.
First Day of Poisoning.

Groups	Conc. of ANTU in food, %	Avg age at start, days	Avg wt at start, g	Avg daily food intake last 10 days on stock diet, g	Avg intake of ANTU food g	Avg intake of ANTU mg	Avg intake of ANTU mg/kg	Avg intake of ANTU Toxic units*	Results
I	10	228	313	13	1.0	100	311.3	62	All 6 died.
II	2	—	272	15	2.1	42	153.8	31	" 10 "
III	1	220	293	13	1.2	12	40.2	8	" 6 "
IV	0.1	205	313	13	2.2	2.2	7.2	1.4	" 6 "
V	0.05	100	265	13	3	1.5	5.8	1.2	" 6 survived
VI	0.01	101	255	13	9	0.9	3.5	0.7	" 6 "
VII	0.005	103	268	13	10	0.5	1.8	0.36	" 6 "
VIII	0.001	82	232	12	12	0.13	0.6	0.12	" 9 "
IX	0.0005	89	227	12	12	0.06	0.3	0.05	" 9 "

*5 mg per kilo body wt.

It is likely that the effects appeared no sooner when rats ingested the 10% concentration than when they ingested the 0.1% concentration, since 5 to 10 minutes appears to be the minimum time required for the poison to become absorbed and to take effect. This explains why the rats ate approximately the same amounts of each of the 4 highest concentrations of ANTU-poisoned food. The amount of ANTU derived from the food with the 0.05% concentration may have made the rats ill in approximately the same time, but the effect probably was not severe enough to stop them from eating at once, as was true with the 4 higher concentrations. Undoubtedly, therefore, the rats exposed to the 5 highest concentrations ate only one meal, while the rats on the lower concentrations, with a food intake which ranged from 9 to 12 g per day, must have eaten at least 3 meals during the 18-hour period.

Development of Tolerance and Refusal. After having survived the first day of poisoning, no rat from any of the 5 remaining groups died during the rest of the 14-day period. At the end of this period, however, when the rats of all groups were offered the 2% ANTU food, only those of Groups VIII and IX, which had previously received the 2 lowest concentrations of ANTU, 0.001% and 0.0005% respectively, died. All of the rats of the other 3 groups survived. Fig. 1D shows a typical record of one of the rats of Group IX. During the 14-day period on the poisoned food this rat continued to eat just as much as it had eaten during the previous 10-day period on the unpoisoned stock diet. When placed on the 2% ANTU diet it ate only 1 g and died. That this amount was smaller than the average intake of previously unpoisoned rats of Group II, which received the 2% ANTU diet, indicates that the rat may have developed a slight refusal; that it died indicated that it had not developed a definite tolerance.

Fig. 1C gives a record which is typical for the rats of the 2 groups, VI and VII, which received diets with the 0.01 and 0.005% concentrations of ANTU respectively. During the 14-day period on the poisoned food this rat continued to eat almost as much as it had previously eaten of the stock diet. When given access to the 2% ANTU food it showed a total refusal for 2 days. Then on the 3rd, 4th, and 5th days, it ate large amounts (over 65 toxic units on one day) and survived. This rat had developed a refusal and also a very marked tolerance during the 14-day period on the poisoned food. At the end of the following 16-day period on the unpoisoned stock diet when the rat was given a choice for only one day of the 2% ANTU diet and of the unpoisoned stock diet, it ate less than 1 g of the poisoned food and died. This indicates that a slight refusal may have remained at this time, but that most of the tolerance had disappeared.

Fig. 1B gives a typical record of the rats of Group V, which received the 0.05% ANTU food for the 14-day period. On the first day of the 14-day period this animal ate 3 g of the poisoned food, which apparently sufficed to make it sick but not to kill it, for on the next day it did not eat any food at all. Each day thereafter, however, it ate progressively more until, toward the end of the period, its intake reached its normal level again, showing that during this period the rat had developed a definite tolerance. When on the 15th day it was given the 2% ANTU food it ate 1 g (20 toxic units of ANTU) and on the 4 succeeding days ate fairly liberal amounts with impunity. Thus this rat appeared to have developed a slight refusal and a very great tolerance.

Its tolerance continued for a longer time than did the tolerance of the rat whose chart is shown in Fig. 1C. Fig. 1B shows further that after 16 days on the unpoisoned stock diet, this rat ate 2 g of the unpoisoned food and 1 g of the poisoned food but showed no ill effects. It was returned to the unpoisoned stock diet for a 13-day period. At the end of this time it ate 3 g, which is more than the average intake of the same ANTU food of previously unpoisoned rats. Thus at this time it showed no definite refusal or tolerance, and died.

Table II summarizes for the 5 surviving groups of rats the results of the observations made for the 14 days during which they re-

TABLE II.
Fourteen Days on ANTU Diet.

		0-14 day period on ANTU diet					15th day—(2% with diet)			
		Avg daily intake of ANTU food	Avg daily intake of ANTU		Avg total intake of ANTU		Avg ANTU food intake	Avg ANTU intake		
Group	Conc. of ANTU in food %	g	mg	Toxic units	mg	Toxic units	g	mg	Toxic units	Results
V	0.05	7.0	3.40	2.82	48.70	39.6	1.0 (5 rats)*	20.0	18.0	All survived
VI	0.01	10.7	1.04	0.82	14.58	12.0	1.0 (2 rats)†	20.0	16.5	" "
VII	0.005	12.9	0.63	0.46	8.93	7.3	1.0 (2 rats)‡	20.0	13.1	" "
VIII	0.001	13.2	0.13	0.10	1.58	1.28	2.4	48.8	36.9	" 9 died
IX	0.0005	12.6	0.06	0.05	0.75	0.62	1.2	24 4	19.3	" 9 "

* One rat did not eat a measurable amount.

† Four rats did not eat measurable amounts.

‡ Four rats did not eat measurable amounts.

TABLE III.
2% Concentration of ANTU—5-Day Period (15th to 19th Days Inclusive).

Group	Avg daily intake of ANTU food g	Avg daily intake of ANTU mg	Avg daily intake of ANTU Toxic units	Total amt of ANTU in 5-day period Toxic units	Results
V	2.2	41.3	39.5	197.5	All 6 survived
VI	0.6	12.6	11.1	55.4	” 6 ”
VII	1.4	28.0	20.1	108.0	” 6 ”

ceived their varying concentrations of ANTU food; it also gives the records for the 15th day, on which they received the 2% ANTU food.

The rats of Group V, which received the 0.05% ANTU diet, ate an average of 7 g, or only slightly more than half of the amount that they had previously eaten of the stock diet. Most of the animals of this group ate very small amounts during the first few days, and almost normal amounts during the last few days of the 14-day period. For the entire period the total amount of ANTU ingested averaged 48.7 mg or 39.6 toxic units, which indicated that a high tolerance had been developed. When given access to the 2% ANTU food, one rat did not eat a measurable amount on the first day while the other 5 ate an average of 1 g, less than half as much as the previously unpoisoned rats ate of this concentration. Their ANTU intake averaged 18.0 toxic units. All 6 rats survived.

During the initial 14-day period the rats of Groups VI and VII, which received the 0.01 and 0.005% concentrations of ANTU, ate only slightly less than they had previously eaten of the plain diet. When given access to the 2% ANTU food most of these rats showed a very strong refusal. Four of the 6 rats of Group VI and 4 of the 6 rats of Group VII completely refused the diet on the first day. The ANTU intake on the first day for the 2 remaining rats of each of the 2 groups averaged 16.5 and 13.1 toxic units respectively. All of the rats of these 2 groups survived.

During the initial 14-day period the rats of Groups VIII and IX ate just as much of the ANTU food as they had previously eaten of the plain diet. When given the 2% ANTU diet they ate almost as much as previously unpoisoned rats ate of the same concentration. Thus, they showed little or no refusal. They ingested 36.9 and 19.3 toxic units respectively and died.

These results show that the daily ingestion over a 14-day period of ANTU in amounts as small as 0.63 mg or 0.46 toxic units sufficed to establish a definite tolerance and refusal.

The high tolerance established in these rats is shown further in Table III, which gives for the 3 remaining groups of rats, V, VI, and VII, the intake records for the 5-day period on 2% food. This daily intake of ANTU averaged 39.5, 11.1 and 20.1 toxic units respectively, while the total intake for the 5-day period averaged 197.5, 55.4 and 108.0 toxic units. None of the rats died. Thus, the rats of these 3 groups developed a very strong tolerance during the 14-day period on their respective concentrations of ANTU food.

Duration of Tolerance and Refusal. Returning the rats of the 3 remaining groups, V, VI, and VII, to the plain stock diet for a 16-day period caused the tolerance and refusal of some of the rats to disappear. Table IV, which summarizes the results, gives the intake of poisoned and of unpoisoned food on the day following the end of the 16-day period; also a record of the mortality. The intake of the plain stock diet averaged 2.0, 1.3 and 2.5 g, respectively, while the intake of the poisoned food averaged 1.6, 1.3 and 2.0 g. One rat in Group V and 2 in Group VII did not eat measurable amounts of the ANTU-poisoned food. The intake of ANTU averaged 25.6, 19.5 and 27.4 toxic units respectively for the 3 groups. All of the rats of Group V survived, while only one of Group VI and 2 of Group VII survived. Thus, the rats which previously had been on the 0.01 and 0.005% concentrations of

TABLE IV.
2% Concentration of ANTU—36th Day (16 Days after Last ANTU).

Group	Avg intake of unpoisoned food g	Avg intake of ANTU food g	Avg intake of ANTU			Results
			mg	mg/kg	Toxic units	
V	2.0	1.6 (5 rats)*	32.0	128.3	25.6	All 6 survived
VI	1.3	1.3	26.6	97.4	19.5	5 died, 1 survived
VII	2.5	2.0 (4 rats)†	40.0	138.0	27.4	4 ” 2 ”

* One rat did not take a measurable amount.
† Two rats did not take measurable amounts.

TABLE V.
2% ANTU—50th Day.

Group	No. of rats still alive	Avg intake of ANTU food g	Avg intake of ANTU			Results
			mg	mg/kg	Toxic units	
V	6	0.18	6.6	24.8	5.0	All 6 died
VI	1	1.0	20.0	69.9	14.0	Died
VII	1	0.75	15.0	52.8	10.6	”
	1	0.5	10.0	33.3	6.6	Survived

ANTU had lost much of their tolerance as well as their refusal. The rats of Group V which had previously received the 0.05% concentration still maintained a high tolerance.

Table V shows that after another period (13 days) on the stock diet the rats of Group V died when they were placed on the 2% ANTU diet. They had lost most of their tolerance. The rats of this group ingested 5.0 toxic units on the average. The small food intake of these rats, 0.18 g, showed that they still had a strong refusal response. The one surviving rat of Group VI ate 1 g, or 14.0 toxic units of ANTU and died. One of the 2 surviving rats of Group VII ate 0.75 g or 10.6 toxic units and died, while the other ate 0.5 g or 6.6 toxic units and survived.

This last rat was returned to the stock diet and exposed again to the 2% ANTU food for one day. It ate scarcely a measurable amount. That the failure to kill this animal depended largely on the presence of a strong refusal response rather than on the presence of a marked tolerance is shown by the fact that forced feeding with a stomach tube a few days later with 30 mg per kg body weight, or 6 toxic units, promptly killed it.

Discussion. The results of these experiments show that the domestic Norway rat readily accepts ANTU-poisoned food in concentrations at least as high as 10%. They show further that a sharply defined boundary exists between the concentrations of ANTU-poisoned food that are lethal and sublethal. All animals that were offered food poisoned with concentrations of 0.1% died, while all animals that ate concentrations of 0.05% survived. The minimum lethal concentration must lie between these 2 concentrations.

The results brought out an equally sharply defined boundary between concentrations of ANTU-poisoned food which did and did not produce a tolerance or refusal. Thus, all of the rats which had received ANTU-poisoned food in a concentration of 0.0005% survived, while all of the rats which received the next lower concentration (0.001%) died when exposed to the 2% concentration of ANTU food. The lowest concentration at which ANTU-poisoned food will produce a tolerance or refusal, or both, must, therefore, lie between these 2 concentrations.

These experiments show that in the 14-day period the rats on the 0.05% concentration of ANTU-poisoned food developed a very great tolerance. The frequent eating of small amounts of poisoned food during the day may account for the ability of the rats to withstand such great amounts of poison. These experiments show also that the tolerance disappeared quite rapidly; in many instances in 16 days; in all instances in 30 days.

The physiological basis for the development of this tolerance remains unknown. ANTU poisoning increases the permeability of the lung capillaries, and as a consequence produces a great pulmonary edema and pleural effusion. The lung edema is apparently the cause of death.[1] The development of tolerance must therefore produce some changes in the effects produced by the poison in the lung capillaries.

It is noteworthy that some animals have a natural tolerance to very large amounts of alpha-naphthyl thiourea.[2] Thus, rhesus monkeys have survived doses of 3500 mg per kg body weight by stomach tube, which is 700 times the amount required to kill domestic Norway rats; likewise chickens have survived doses of 2500 mg per kg body weight. Guinea pigs, rabbits and most wild rodents, including Alexandrine rats, have a high tolerance. Of the animals tested so far dogs and pigs[3] are the only animals besides Norway rats which do not show this tolerance. Their LD_{50} lies between 25-35 mg per kg body weight which is still much higher than that for the strain of rats used in the present studies.

Domestic Norway rats from colonies in some laboratories and wild Norway rats, recently trapped from the streets, show a high resistance to the parent compound thiourea, while the rats from our colony die after the ingestion of minute doses.[4] It was found that while 640 mg per kg body weight was required to kill the rats of the Long-Evans strain from Dr. Astwood's colony in Boston, 1830 mg per kg body weight was required to kill recently trapped wild Norway rats. Recently trapped wild Alexandrine rats *(Rattus rattus alexandrinus)* showed the same high resistance.

The resistance to thiourea poisoning does not seem to be a natural tolerance since it is markedly influenced by diet. Thus feeding our stock diet for 1-6 months reduced the LD_{50} of the rats from Dr. Astwood's colony to 44 mg per kg body weight,[4] and feeding experimental rats of the hooded (Rowett) strain a straight oatmeal diet for 4 weeks reduced the amount of thiourea at which a 100% mortality was obtained from 200 mg to 20 mg.[5] Likewise feeding adult wild Norways on our stock diet reduced the LD_{50} from 1830 mg per kg body weight to below 500 mg per kg body weight in 9 months.[6]

These experiments show also that after eating the ANTU-poisoned food, even in a very low concentration, the rats developed a marked refusal to eat food that was poisoned with definitely lethal concentrations. The lower concentrations, which apparently did not produce such a marked tolerance, developed a greater refusal. In some animals the refusal was much reduced after 16 days and practically absent after 30 days. However, in one animal it remained very strong after 36 days and gave no sign of diminishing.

The basis for this refusal response has not been determined. It could depend on association of ill effects with the taste of ANTU, or with the smell. ANTU has a moderately bitter taste to some human subjects and therefore it probably has a bitter taste to rats also. Observations made on rats as they ate ANTU mixtures in concentrations as high as 20% demonstrated that its taste cannot be extremely unpleasant and bitter to them. The slight bitterness of the ANTU may however suffice as a cue for the refusal. In the form used in these experiments ANTU had an extremely faint odor. This odor, scarcely detectable by human subjects, may be readily detected by rats, since their olfactory sensitivity probably is much greater than that of human beings.[§] The rats may detect it in the surrounding air since it floats readily in air, and can then be detected by its taste rather than by its smell. Sectioning of taste nerves and removal of the olfactory bulb in rats with a strong refusal reaction should differentiate the part played by taste and smell in the refusal response.

---

[3] Anderson, W. A., and Richter, C. P., *Vet. Med.*, 1946, **41**, 302.

[4] Dieke, S. H., and Richter, C. P., *J. Pharmacol.*, 1945, **83**, 195.

[5] Landgrebe, F. W., and Morgan, T. N., *Nature*, 1946, **157**, 22.

[6] Dieke, S. H., and Richter, C. P., unpublished data.

[§] Wild Norway rats may have an even greater sensitivity than domestic rats.

These experiments show that when rats are exposed to ANTU, which they probably taste only faintly, they are able to protect themselves by a rapid development of both a tolerance and a refusal. It is unlikely that rats ever encounter ANTU or closely allied thiourea compounds in the wild state, but the principle involved in the development of tolerance and refusal probably holds equally true for other poisons.

Summary. 1. Adult domestic Norway rats were found to accept ANTU when mixed with the stock diet in concentrations at least as high as 10%. In all concentrations from 0.1% to 10% inclusive the ANTU-poisoned food killed 100% of the rats. Lower concentrations of ANTU-poisoned food gave a 100% survival.

2. When fed sublethal concentrations of ANTU food from 0.05% to 0.005% inclusive for 14 days, the rats developed a marked tolerance and refusal. Lower concentrations failed to produce either.

3. In some rats the tolerance disappeared at the end of 16 days and was almost imperceptible in all of the rats at the end of 30 days.

4. The refusal decreased after 16 days, though in some animals it was definitely present even at the end of 30 days.

15604

Further Studies on Relationship Between Activity and Constitution in the Cardiac Glycoside Series.*

ROBERT A. LEHMAN.

From the Department of Therapeutics, New York University College of Medicine.

In a recent report[1] various glycosides and genins were compared with respect to their ability to produce atrioventricular block in the isolated embryonic chick heart, and it was possible to suggest certain conclusions as to the relationship between chemical structure and cardiac activity. The technic has been described in detail and a procedure for estimating the precision of the results has been proposed.[2] A number of additional glycosides and related compounds have now been studied and the results are given in Table I. In general, each compound was compared directly with a closely related reference material and indirect comparisons were computed from previously published activity ratios.

For practical reasons it is difficult to study the interrelationships of the glycosides in man. Hence, in comparing these drugs on an artificial preparation it is necessary to inquire into the significance of the results with respect to the observed clinical potencies. Comparison of the oral therapeutic dose for 3 well known drugs with the results obtained by bio-assay showed a direct correlation with the embryonic heart data but divergent results with respect to the cat assay.[1] Thus, the ratios of the human daily maintenance doses for digitoxin, digoxin, and lanatoside C were 1:3:7; the concentration ratios for atrioventricular block in the isolated heart, 1:3:4.5; while for the intravenous lethal doses in the cat the ratios were 1:0.7:0.45. Each of these ratios has been computed on

---

* The author gratefully acknowledges a grant from the John and Mary R. Markle Foundation, under which this study was carried out. Many of the glycosides used were made available through the kind cooperation of Dr. K. K. Chen. The technical assistance of Miss Alice Sullivan is greatly appreciated.

[1] DeGraff, A. C., Paff, G. H., and Lehman, R. A., *J. Pharmacol.*, 1941, **72**, 211.

[2] Lehman, R. A., and Paff, G. H., *J. Pharmacol.*, 1942, **75**, 207.

TABLE I.
Comparison of Glycosides and Derivatives.

Compound	Molecular wt	Reference compound	Potency for embryonic chick heart in terms of reference compound ± standard error (on a molar basis)	Potency by cat assay in terms of reference compound (on a molar basis)*
Convallatoxin	550	Cymarin	1.00 ± 0.12	1.40[3]
Strophanthidin-β-d-glucoside	566	”	0.96 ± 0.037	1.25[3]
Strophanthidin-tetra-acetyl-β-d-glucoside	734	Strophanthidin	1.20 ± 0.098	0.31[4]
Strophanthidin-3-acetate	462	”	1.20 ± 0.074	1.99[3]
Strophanthidin-3-n-caproate	518	”	1.45 ± 0.15	1.94[3]
Periplocymarin	534	Cymarin	1.09 ± 0.12	0.70[3]
Thevetin	867	Digitoxin	0.040 ± 0.0030	0.42[3]
Ouabain	584	”	0.121 ± 0.009	2.14[3]
			0.144 ± 0.012	
Gitalin (amorphous)	668	”	0.550 ± 0.040	0.38[5]
			0.575 ± 0.036	
α-β-angelica lactone	98	”	0.0021 ± 0.00012	—[6]
β-γ-angelica lactone	98	”	0.0013 ± 0.00011	—[6]
Cerberin	522	”	1.64 ± 0.17	1.51[3]

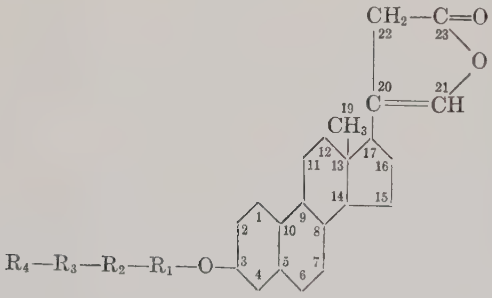
* Values in this column have beeen computed from the lethal dose in the literature for the individual glycosides and do not represent direct comparisons as do the embryonic chick heart data.

TABLE II.
Summary of Factors Which Modify the Inherent Potency of the Glycosides as Estimated by Various Methods.

	Latent period	Absorption from the gastrointestinal tract	Ability of the heart to remove the glycoside from the blood stream in competition with dissipation processes
Oral maintenance dose in man	Little or no significance	Importance questionable; there has been no direct estimation of the rate and completeness of absorption.	Probably the determining factor.
Lethal dose by intravenous infusion in the cat within 60 to 90 min.	Very important; the longer the latent period the less potent the drug appears.	Not involved.	Relatively unimportant because of the rapid introduction of the drug into the blood stream and the high concentration to which the heart is subjected.
A-V block concentration in the isolated embryonic chick heart	No significance.	” ”	Removal of drug from the surrounding medium probably very important; dissipation is not involved.

a molar basis but the discrepancy would be the same in terms of weight. The explanation for this is probably to be found in the relative importance of the factors which modify the inherent potency of these drugs when estimated by different procedures. These factors are summarized in Table II which serves to emphasize the incompleteness of our knowledge and suggests that the isolated heart method may at least measure one of the more important factors which determine the oral dose in man. On the other hand, it is evident that the cat assay gives major emphasis to a factor which is of no significance in man by this route of administration. If a table of cat unit potencies be examined, such as that in a recent review by Chen,[3] the shortcomings of this method of evaluation become immediately apparent. Thus, digitoxin appears to have the same potency as strophanthidin (0.325 ± 0.011 and 0.325 ± 0.023 mg per kg) and to be only slightly more potent than its own genin (0.459 ± 0.036). By the chick heart method digitoxin was found to be 9.6 times strophanthidin and 8.9 times digitoxigenin.[1] Similar comparisons are given below.

Results. The basic skeleton of the glycosides is



The following observations are based on the data of Table I:

The Substituent on C-3. In the naturally occurring glycosides a carbohydrate residue is attached to the ring through the OH group on C-3. This residue may be a single desoxyhexose or made up of 2, 3 or 4 sugar units. It is generally recognized that such a substituent is essential to secure the "cumulative" property characteristic of these drugs. "Cumulative" is here used in the sense of the earlier work on the relative ease of washing the glycosides out of the heart. As suggested in Table II, the extent to which this property is taken into account in the estimation of potency depends upon the method used. The embryonic heart method appears to give considerable weight to this factor as indicated by a 9- to 37-fold increase in potency when various glycosides were compared with their genins. However, Chen has concluded that, "Different sugars may intensify the cardiac action to a different degree."[3] This does not appear to follow in the isolated heart so long as only a single sugar unit is involved. Strophanthidin cymaroside (Cymarin), strophanthidin rhamnoside (Convallatoxin) and strophanthidin-β-d-glucoside (synthetic) are identical in activity within the limits of experimental error. As more sugar units are added to the molecule, however, the potency decreases somewhat. Complete blocking of the OH groups of the sugar residue such as by acetylation of strophanthidin-β-d-glucoside to give the tetra acetyl derivative, yields a compound with little greater activity than the parent genin.

Attempts have been recently made to substitute other groups at C-3. A theophylline derivative of scillaridin was found to be active but the compound was never adequately characterized.[4,5] Many synthetic esters of strophanthidin have been studied, especially by Chen[4,9] and Gold[10] and their coworkers. Results are included in Table I for the acetate and caproate which were found to be slightly more potent than strophanthidin. This agrees with Gold's findings that their action in man by oral administration is very weak, which he considers to be due to poor absorption but concedes, "There is the possibility that more might have been absorbed but the very rapid elimination kept pace with a relatively slow absorption."

Other Structural Relationships. (1) Periplocymarin and cymarin, which differ from each other only in having respectively a —CH_3 and a —CHO group on C-10, are of identical potency. This is in fair agreement with cat assay results if allowance is made for experimental error.

---

[3] Chen, K. K., *Annual Review of Physiology*, Annual Reviews, Inc., Stanford University, 1945, Vol. VII, p. 677.

[4] Chen, K. K., and Elderfield, R. C., *J. Pharmacol.*, 1942, **76**, 81.

[5] *New and Non-Official Remedies*, American Medical Association, 1946, p. 329.

[6] Chen, K. K., Steldt, F. A., Fried, J., and Elderfield, R. C., *J. Pharmacol.*, 1942, **74**, 381.

[7] Riseman, J. E. F., and London, S. B., *Science*, 1940, **92**, 384.

[8] DeGraff, A. C., and Lehman, R. A., PROC. SOC. EXP. BIOL. AND MED., 1941, **46**, 36.

[9] Steldt, F. A., Anderson, R. C., Maze, N., and Chen, K. K., PROC. SOC. EXP. BIOL. AND MED., 1943, **53**, 198.

[10] Gold, H., Otto, H. L., Modell, W., and Halpern, S. L., *J. Pharmacol.*, 1946, **86**, 301.

(2) A daily oral dose in man of 1.3 to 3.7 mg has been suggested for thevetin[11] as compared with 0.1 to 0.2 mg for digitoxin[12] or an average molar ratio of about 1/15. In the isolated heart thevetin is 1/25 as potent as digitoxin, which is in much better agreement than is the cat assay for which the ratio is about 1/2. The genins corresponding to these 2 drugs are stereo-isomeric and different triosides are attached at C-3. The influence of the stereochemical arrangement may be judged if it is assumed that this difference in the sugars is of minor significance.

(3) Ouabain is one-eighth as active as digitoxin. In man, ouabain is considered an unreliable drug by the oral route but it has never been determined with certainty whether this is due to poor absorption, rapid dissipation or failure to "cumulate." Ouabain is twice as potent as digitoxin in the cat.

(4) Amphorous gitalin from the water-soluble fraction of Digitalis purpurea has about half the potency of digitoxin by either method. Stroud and coworkers[13] found the daily oral dose of gitalin to be approximately 0.27 mg, which gives a molar ratio to digitoxin of about 0.49.

(5) The 2 angelica lactones show an activity 1/800 to 1/500 that of digitoxin. Furthermore, their effect on the heart is qualitatively different from the glycosides in that ventricular arrest frequently appears before and instead of atrioventricular block. This contributes further evidence that the lactone ring is only part of the structural configuration necessary for the typical effects of the cardiac glycosides. These compounds are not considered to have digitalis-like action in the cat.

(6) Cerberin is a crystalline glycoside of uncertain structure, which, like gitalin, has about the same ratio to digitoxin by either method.

Summary. The potency of a number of glycosides and related compounds for the embryonic chick heart has been reported and various implications suggested. As a means of studying the relationship of chemical constitution and physiologic action, the embryonic heart method appears to offer definite advantages over methods employing intravenous administration to intact animals.

---

[11] Middleton, W. S., and Chen, K. K., *Am. Heart J.*, 1936, **11**, 75.

[12] Gold, H., Kwit, N., and Cattell, McK., *J. Pharmacol.*, 1940, **69**, 177.

[13] Stroud, W. D., Livingston, A. E., Bromer, A. W., Vander Veer, J. B., and Griffith, G. C., *Ann. Int. Med.*, 1934, **8**, 710.

15605

Failure of Tyrothricin in the Treatment of Experimental Syphilis of Rabbits.

John A. Kolmer and Anna M. Rule.

From the Research Institute of Cutaneous Medicine, Philadelphia, Pa.

From the standpoint of antibiotic therapy the possible therapeutic effectiveness of tyrothricin in the treatment of experimental syphilis of rabbits is not without interest. Owing to its toxicity by intravenous injection, however, largely due to hemolytic activity, the compound has not been generally employed in the treatment of experimental infections of the lower animals by this route of administration.

In these experiments a 2% alcoholic solution of tyrothricin (Parke-Davis Co.) was employed, suspensions being freshly prepared by diluting 1 cc with 99 cc of sterile distilled

TABLE I.
Toxicity of Tyrothricin for Rabbits by Intravenous Injection.

No.	Dose per kg g	Survival: days													
		1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	0.0002	—	—	—	—	—	—	—	—	—	—	—	—	—	—
2	0.0002	—	—	—	—	—	—	—	—	—	—	—	—	—	—
3	0.0004	—	—	—	—	—	—	—	—	—	—	—	—	—	—
4	0.0004	—	—	—	—	—	—	—	—	—	—	—	—	—	—
5	0.0006	—	—	—	—	—	—	—	—	—	—	—	—	—	—
6	0.0006	—	—	—	—	—	—	—	—	—	—	—	—	—	—
7	0.0008	—	—	—	—	—	—	D							
8	0.0008	—	—	—	—	—	—	—	—	—	D				
9	0.001	—	—	—	D										
10	0.001	—	—	—	—	D									

water (0.0002 g per cc). Normal adult rabbits were given varying amounts by intravenous injection as shown in Table I; with the tyrothricin employed the maximum tolerated dose was about 0.0006 g per kg of weight.

Sixteen rabbits were inoculated intratesticularly with the Nichols-Hough strain of *T. pallidum*. In from 4 to 6 weeks all developed acute syphilomas with positive darkfield examinations. Four rabbits were kept as untreated controls while the remaining 12 were given daily intravenous injections of 0.5 cc of 1:100 suspensions of tyrothricin per kg of weight over a period of 15 days. This dose corresponded to 0.0001 g per kg, being equivalent to about one-sixth of the maximum single tolerated dose. Clinical records and darkfield examinations for *T. pallidum* were made at daily intervals.

All animals survived with apparently no clinical evidences of toxicity. During and at the end of the period of treatment none showed any clinical evidences of healing of the testicular lesions as compared with the untreated controls and all 16 animals showed persistently positive darkfield examinations. Under the circumstances lymph node transfers were regarded unnecessary.

Summary. The maximum single tolerated dose of tyrothricin for adult rabbits by intravenous injection was about 0.0006 g per kg. Fifteen intravenous injections of 0.0001 g per kg, at daily intervals (totalling 0.0015 g per kg) were completely ineffective in the treatment of acute testicular syphilis of rabbits.

15606

Failure of Penicillin and Streptomycin in the Prophylaxis and Treatment of Experimental Vaccinia of Rabbits.

JOHN A. KOLMER AND ANNA M. RULE.
From the Research Institute of Cutaneous Medicine, Philadelphia, Pa.

Andrews and his colleagues[1] have reported that penicillin is ineffective in the treatment of vaccinia and Parker and Diefendorf[2] have found that the compound was without effect on the multiplication of the virus in the Rivers-Li culture medium and in the intact chick embryo. The activity of streptomycin on the virus has not been reported upon but the results of experiments herein briefly summarized, employing both compounds in the prophylaxis and treatment of experimental vaccinia of rabbits, have shown that they are completely ineffective. Under the circumstances any value that the compounds may possess in the treatment of severe generalized vaccinia or variola of human beings would probably depend upon the prevention or control of secondary staphylococcal or streptococcal infections of the lesions.

---

[1] Andrews, C. H., *et al.*, *J. Path. and Bact.*, 1943, **55**, 173.

[2] Parker, R. F., and Diefendorf, H. W., PROC. SOC. EXP. BIOL. AND MED., 1944, **57**, 351.

In these experiments the prepared abdominal skins of adult rabbits were scarified and the areas inoculated with 1:500 dilutions of stock crude vaccinia virus in 50% glycerin.*

Immediately after inoculation 4 rabbits were given an intravenous injection of 1000 units of penicillin† per kg of weight, followed by a second injection 4 hours later. Thereafter, injections were given at 9 a.m., 1 p.m. and 4 p.m. daily for 5 days in succession, totalling 17 doses or 17,000 units per kg. Four additional rabbits were treated in the same manner with 10,000 units of streptomycin‡ in saline solution by intramuscular injection, totalling 17 doses or 170,000 units per kg. All animals, including 4 untreated controls, developed vaccinal lesions on or about the 5th day after inoculation and were kept under observation for a total of 15 days following inoculation. None of the treated animals showed the slightest activity on the part of penicillin and streptomycin in the prophylaxis of vaccinia as compared with the untreated controls.

Twelve additional rabbits were inoculated in the same manner. Four were kept as untreated controls. Treatment of the others was instituted on the 5th to 6th days after inoculation at which time vaccinal lesions were present. Four rabbits were given penicillin and 4 streptomycin in exactly the same manner and in the same dosages as described above. All animals were kept under observation for a total of 20 days following inoculation. None of the treated animals showed the slightest clinical improvement or suppression of developing lesions, as compared with the untreated controls.

Summary. Penicillin in dose of 1000 units per kg by intravenous injection for a total of 17 doses (17,000 units per kg) was completely ineffective in the prophylaxis and treatment of experimental vaccinia of rabbits. Streptomycin in dose of 10,000 units per kg by intramuscular injection for a total of 17 doses (170,000 units per kg) was likewise completely ineffective in the prophylaxis and treatment of experimental vaccinia of rabbits.

---

* Kindly supplied by Dr. B. Hampil of the Sharp and Dohme Co., Glenolden, Pa.

† Commercial penicillin (Lot No. 45072102) kindly supplied by the Commercial Solvents Corp.

‡ Kindly supplied by the Abbott Laboratories, North Chicago, Ill.

15607

Survival of Rats Given Methionine* Before and After Thermal Injury.

MILES D. McCARTHY AND RICHARD E. BODKIN. (Introduced by I. S. Ravdin.)

From the Harrison Department of Surgical Research, Schools of Medicine, University of Pennsylvania, Philadelphia.

The beneficial action of *dl*-methionine in combating selenium poisoning[1] and various types of hepatic injury[2-4] has been well established. Methionine has also been shown to have a protein-sparing action when fed to normal dogs on a low protein diet[5] and following thermal burns in rats.[6] We have

---

* The methionine used in these experiments was supplied by Wyeth, Incorporated.

[1] Lewis, H. B., Schultz, J., and Gortner, R. A., *J. Pharm. and Exp. Therap.*, 1940, **68**, 292.

[2] György, P., *Am. J. Clin. Path.*, 1944, **14**, 67.

[3] Miller, L. L., Ross, J. F., and Whipple, G. H., *Am. J. Med. Sc.*, 1940, **200**, 739.

[4] Beattie, J., *Lancet*, 1944, **2**, 787.

[5] Miller, L. L., *J. Biol. Chem.*, 1944, **152**, 603.

[6] Croft, P. B., and Peters, R. A., *Lancet*, 1945, **1**, 266.

TABLE I.
The 48-Hour Survival of Burned Rats Given 160 mg of Methionine in Aqueous Solution 2 Hours Pre-burn.

Exp. No.	Date	Methionine treated		Water controls		Untreated		Surface burned %
		Burned	Survived	Burned	Survived	Burned	Survived	
62	9-26-45	3	3	4	0	4	2	30-33
68	10-13	4	4	3	2	4	2	26-30
72	26	4	4	4	4	4	3	25-29
74	27	2	1	3	2	4	1	25-29
78	11-17	6	6	3	2	4	3	28-31
80	16	2	1	4	2	2	2	25-29
82	21	3	3	1	0	1	0	30-34
84	24	2	2	4	1	4	1	28-30
108	6-11-46	8	7	8	1	8	2	29-31
Totals		34	31	34	14	35	16	
% survival			91		41		46	

TABLE II.
The 48-Hour Survival of Burned Rats Given 320 mg of Methionine in Aqueous Solution Post-burn.

Exp. No.	Date	Methionine treated		Water controls		"Tubed" controls		Untreated		Surface burned %
		Burned	Survived	Burned	Survived	Burned	Survived	Burned	Survived	
Time of therapy post-burn—1 and 5 hr.										
104	12-13-45	8	7	8	4					29-32
105	14	8	8	8	3					28-32
106	17	4	1	4	2	6	1	2	0	28-32
107	18	10	3	9	9	4	1			28-32
Totals		30	19	29	18	10	2	2	0	
% survival			63		62		20		0	
Time of therapy post-burn—0.5 and 4 hr.										
110	6-11-46	4	1	4	2			6	2	29-31
Time of therapy post-burn—immediately and 3 hr.										
111	6-14-46	8	5	8	8	8	1			28-31
114	13	8	3	8	2			6	1	30-34
Total of exper. 104-114		50	28	49	30	18	3	14	3	
% survival			56		61		17		21	

tested the effect of the preburn and postburn administration of methionine on the survival rate of rats subjected to a standard burn.

Methods and Results. Normal stock Wistar rats weighing between 190 and 210 g and maintained on an *ad libitum* diet of Purina Dog Checkers supplemented with whole milk were used in these experiments.

In all experiments a group of healthy rats was selected from the stock cages. In the experiments of Series I, the animals were divided into 3 groups. One group was given 160 mg of methionine in 5.0 cc of distilled water orally through a No. 8 French catheter. A second group was given 5.0 cc of distilled water by the same technic. The third group received no treatment. Two hours after the administration of the methionine solution or water all rats in each of the 3 groups were anesthetized with ether and subjected to a standardized back burn[7] in water at 90°C ±0.5° for 23 seconds. The extent of the burned areas of the rats in each experiment was determined by planimetric measurements, as has been previously described.[†] A 91% survival for the methionine-treated rats (Table I) as compared to 41% survival for the water controls and 46% for the untreated controls was obtained. The increased survival of the methionine group compared to either of the control groups is statistically significant. A P value of less than 0.01 was obtained by calculating the X^2 value from the formula of Fisher[8] for 4-fold tables.

---

[7] McCarthy, M. D., *J. Lab. and Clin. Med.*, 1945, **30**, 1027.

As a consequence of the beneficial results obtained in this series of experiments, several experiments were done in which a single dose of 160 mg of methionine in 5.0 cc of distilled water was given to rats one hour following the burn. These experiments showed no difference in survival between the methionine-treated and water-treated animals. Four experiments were then performed in each of which all rats of one group received 2 doses of 160 mg each of methionine in 5.0 cc of distilled water at one and 5 hours following the burn. A second group in each experiment received 5.0 cc of distilled water at the same postburn intervals. In the third and fourth experiments "tubed" control groups were included. The rats in these groups were subjected to the passage of the moistened catheter at the same time intervals postburn that the treated animals received therapy.

Survival percentages from the summarized data of these experiments No. 104, 105, 106 and 107 (Table II) showed no difference in survival between the methionine-treated and the water-treated rats.

Three further experiments were done, No. 110, 111 and 114, in which the same quantities of methionine were given at shorter time intervals postburn. In experiment No. 110, 160 mg of methionine was given at a half-hour and at 4 hours following the burn. No beneficial results from the methionine were obtained. In experiment No. 111, in which "tubed" as well as water controls were used, similar quantities of methionine were given immediately postburn and at 3 hours postburn. In experiment No. 114, 160 mg of methionine in 5.0 cc of distilled water was given intraperitoneally immediately postburn and at 3 hours postburn. Beneficial results were not obtained from the methionine therapy in these latter 2 experiments. The survival percentages of the methionine-treated rats, calculated from the totals of all experiments in which methionine was administered postburn (Table II), was not significantly different from that of the simultaneous water-control rats. However, the difference in the survival rates of both the water and methionine-treated animals compared with either the moistened tube controls or the untreated controls is significant, P = less than 0.01 for the tubed controls and 0.02 for the untreated controls.

Summary. One hundred and sixty mg of methionine administered orally to rats 2 hours before subjecting them to a standardized thermal injury significantly increased the 48-hour survival rate. One hundred and sixty mg of methionine administered to rats orally following thermal injury did not increase their survival rate. Three hundred and twenty mg of methionine administered either orally or intraperitoneally following thermal injury failed to increase the 48-hour survival rate.

Ten cc of distilled water administered orally following thermal injury significantly increased the 48-hour survival rate.

[†] *Ibid.*

[8] Fisher, R. A., *Statistical Method for Research Workers*, London, 1938, Oliver and Boyd, Ltd.

15608

Inhibition of Endochondral Ossification in Pantothenic Acid Deficiency.

BARNET M. LEVY AND MARTIN SILBERBERG.

From the Department of Oral Pathology, Washington University School of Dentistry, and the Snodgras Laboratory, City Hospital of St. Louis, St. Louis, Mo.

Growing mice and rats kept on a diet deficient in pantothenic acid lose or fail to gain weight;[1] in young rats this deficiency causes hypoplasia of the growing cartilage.[2] To our knowledge, however, no detailed studies have been made of the histological changes occurring in the skeleton under this condition.

Material and Methods. Thirty-seven mice of the closely inbred strain C57 black were used. Thirty animals were 4 to 5 weeks old, and 7 were 10 to 12 months old at the beginning of the experiments. The mice were fed a synthetic diet deficient in pantothenic acid *ad libitum* for varying periods of time. Water was available at all times. The ration consisted of

Sucrose	68	%
Vitamin test casein	18	,,
Vegetable oil	10	,,
U.S.P. salt mixture No. 2	4	,,
Cod liver oil	1	,,
Riboflavin	0.5 mg	,,
Thiamin	0.2 ,,	,,
Nicotinic acid	1.0 ,,	,,
Pyridoxine	0.2 ,,	,,
Choline chloride	1.0 ,,	,,
Inositol	100.0 ,,	,,*

Nine growing animals were kept on the deficient diet for 4 to 5 weeks, after which period they were fed Purina Dog Chow, and repair was allowed to take place for varying periods of time. Details concerning the number of animals, their age, sex and time of observation in each group are given in Table I.

Additional mice of the same strain and sexes, but fed the complete synthetic diet, as well as animals kept on the standard diet, served as controls. Ten mg % calcium pantothenate was added to the deficient diet for synthetic control diet.

At the end of the periods, the experimental and control mice were sacrificed; the tibia and femur were removed as a whole, decalcified in 45% formic acid, embedded in paraffin, sectioned and stained with hematoxylin and eosin. The studies were made at the growth zones of the upper tibia and lower femur.

Observations. Gross. After about one week on the deficient diet, the animals began to lose hair, and smooth bald areas appeared on the back or over the rump. This hair loss increased markedly during the experimental period, and the skin became dry and scaly. The weights of the animals are to be found in Column 6 of the table. The mice kept on the deficient diet showed considerable loss of weight. Upon being fed the repair diet, their weight increase was rapid and amounted in some animals to as much as 7 g in 5 days.

Histological Examination. A description of the histological appearance of the growth zones in normal mice (Fig. 1), has been given elsewhere.[3] In growing male mice kept on the deficient diet for one week (Fig. 2), the epiphyseal disk was only half as wide as in the controls. The cartilage cell rows were regular, but they contained only from 6 to 4 inactive columnar and from 3 to one hypertrophic cartilage cells instead of the usual number of 10 columnar and 4 hypertrophic cells. Both cell types were distinctly smaller than ordinarily. The ground substance was more abundant than in the controls. Calcification of the provisional cartilage proceeded at a regular rate. The metaphysis contained few and collapsed capillaries. The primary trabeculae were

---

[1] Morris, H. P., *J. Nat. Cancer Inst.*, 1944, **5**, 115.

[2] Ashburn, L. L., *Pub. Health Rep.*, 1940, **55**, 1337.

* We are indebted to Merck and Company for the liberal supply of vitamins used in this study.

[3] Silberberg, M., and Silberberg, R., *Am. J. Anat.*, 1941, **68**, 691.

TABLE I.

No. of animals	Sex ♂	♀	Age at beginning of exp.	Time fed deficient diet	Time fed repair diet	Mean wt (g) Initial	Final
6	3	3	4-4½ wk.	1 wk.	—	13 ± 4	13 ± 2
6	2	4	4-4½ "	2 "	—	14 ± 3	12 ± 2
9	5	4	4-4½ "	4 "	—	13 ± 4	11 ± 2
3	2	1	4-4½ "	4½ "	5 days	14 ± 3	21 ± 3
2	1	1	4-4½ "	4½ "	2 wk.	15 ± 3	23 ± 1
2	1	1	4-4½ "	4½ "	3 "	15 ± 2	24 ± 1
2	1	1	4-4½ "	4½ "	4 "	16	25
2		2	10-12 mo.	1 "	—	36 ± 3	32 ± 2
1		1	10-12 "	2 "	—	36	36
1		1	10-12 "	4 "	—	36	35
1		1	10-12 "	7 "	—	37	35
2		2	10-12 "	9 "	—	34 ± 1	32 ± 2

much shorter and scarcer than in the controls; they were covered by a discontinuous layer of small and flat spindle cells instead of the usual osteoblastic cell layer. The shaft consisted of small osteocytes; osteoblasts at the inner and outer layers were scanty, and vascularization was less marked than usual.

In the corresponding females (Fig. 3), the changes were less accentuated than in the males. The growth zones were about two-thirds as wide as those of the control female fed the complete synthetic diet. In the single cartilage cell row, 8 or 7 columnar and 3 or 2 hypertrophic cells were counted. They were smaller than in the normal controls, but larger than in the corresponding deficient males. The amount of matrix present was intermediate between the normal and that seen in the deficient males. Similarly, the bony spicules were fewer, shorter and thinner than usual, but they were thicker, longer and more numerous than in the deficient males, and more osteoblasts were found.

In males kept on the deficient diet for 2 weeks, the cartilage cell rows were composed of 5 small columnar cells and one small hypertrophic cell. The matrix had further increased in amount and density, whereas metaphysis and shaft had not changed as compared with the previous stage. After 4 weeks, the structure of the cartilage was not different from that seen after 2 weeks. However, the trabeculae had become still shorter, and they showed some transverse bony links (Fig. 4), indicating a cessation of growth. In healthy untreated animals of this strain, this condition is not seen before the age of 4 to 5 months. Females fed the deficient diet for 2 weeks showed more marked changes than those deficient for one week. The cartilage cell rows were shorter and were composed of 7 or 6 columnar and 3 or 2 hypertrophic cells. After 4 weeks, the single row contained 5 columnar cells, whereas the number of hypertrophic cells remained 3 or 2.

In the animals kept on the deficient diet for 4½ weeks and returned to the standard diet, processes of repair took place rapidly. After only 5 days of refeeding (Fig. 6), the growth zones were as wide and, in some instances, even wider than in the controls; a single row containing from 12 to 10 mitotically proliferating large columnar and 4 large hypertrophic cartilage cells. The matrix was less abundant; calcification of the cartilage and replacement by bone were in good progress. The vascularization of metaphysis and shaft was good. The number and thickness of spicules were the same as seen in young healthy controls, and osteoblasts were plentiful everywhere. The histological pattern resembled that of a rapidly growing control animal 4 to 5 weeks of age. However, at the later stages, in spite of active growth of the epiphyseal cartilage, intensified hyalinization of the matrix indicated some previous damage to the cartilage.

In the adult mice fed the deficient diet, the epiphyseal cartilage was inactive, and in some places epiphyseodiaphyseal union was about to occur. No effect of the defi-

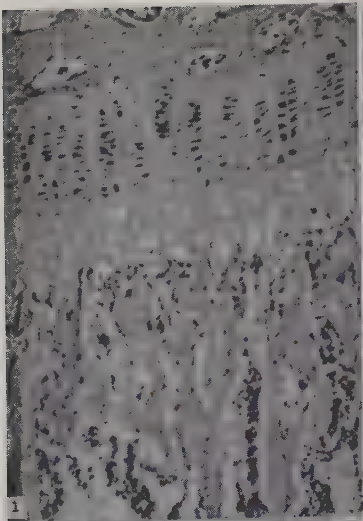
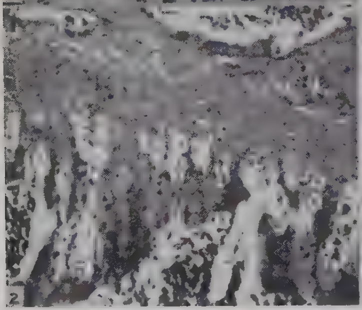
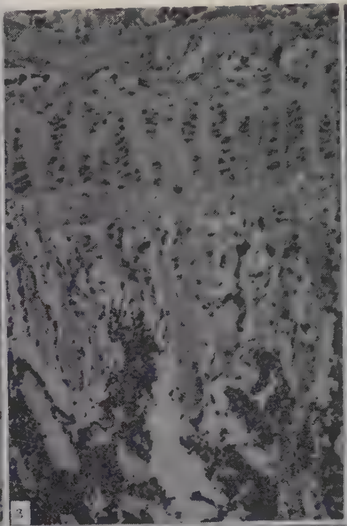
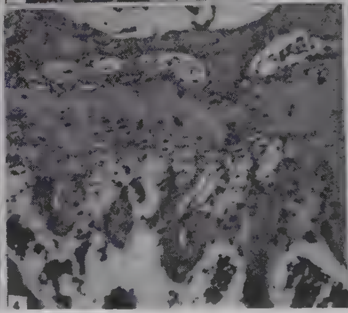
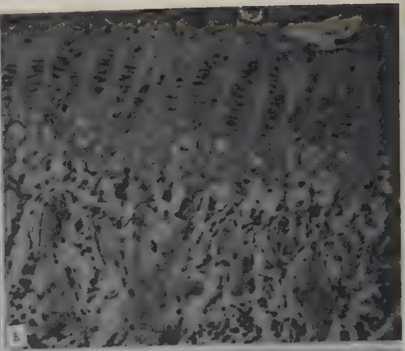
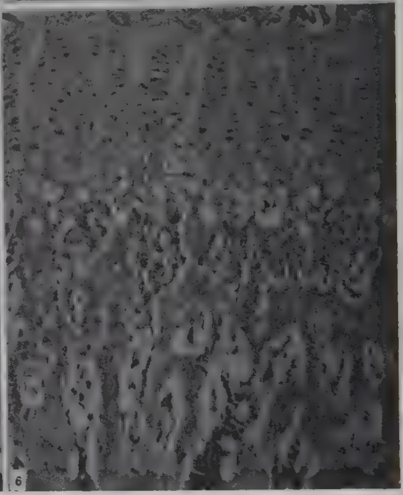







FIG. 1.—Section through the growth zone of the upper tibia of a 6-week-old female mouse of Strain C 57 black fed the synthetic control diet since the age of 4 weeks. Magnification 100×.

The epiphyseal disc is wide and shows regular configuration. The metaphysis contains numerous long trabeculæ separated by bone marrow.

FIG. 2.—Section through the growth zone of the upper tibia of a 6-week-old male mouse of Strain C 57 black fed the deficient diet for one week. Magnification 100×.

The epiphyseal disc is narrowed as compared with Fig. 1, and the subepiphyseal spicules are fewer and shorter than in the control.

FIG. 3.—Section through the growth zone through the upper tibia of a 6-week-old female mouse of Strain C 57 black fed the deficient diet for one week. Magnification 100×.

The epiphyseal disc is narrower than in the control (Fig. 1) but wider than in the deficient male (Fig. 2). The subepiphyseal spicules are decreased in number as compared with the normal control (Fig. 1).

FIG. 4.—Section through the growth zone of the upper tibia of an 8-week-old male mouse of Strain C 57 black fed the deficient diet for 4 weeks. Magnification 100×.

The epiphyseal disc is markedly narrowed in width. The subepiphyseal trabeculæ show transverse bony links.

FIG. 5.—Section through the growth zone of the upper tibia of a 3-week-old female mouse of Strain C 57 black fed the deficient diet for 4 weeks. Magnification 100×.

The epiphyseal plate is wider than in the corresponding male (Fig. 4). There is a suggestion of early transverse bony links uniting the subepiphyseal spicules.

FIG. 6.—Section through the growth zone of the upper tibia of an 8-week-old male mouse of Strain C 57 black fed the deficient diet for 4½ weeks, and subsequently refed the stock diet for 5 days. Magnification 100×.

The epiphyseal disc resembles that of the 6-week-old control animal (Fig. 1).

cient diet was noted in either cartilage, metaphysis, bone marrow or shaft.

Comment. In growing mice of strain C57 black, pantothenic acid deficiency caused an inhibition of endochondral ossification. Proliferation as well as hypertrophy of the cartilage were markedly decreased or, at later stages, completely arrested. Associated with the inhibition of growth was intensified hyalinization of the matrix, but no disturbances in calcification were noted. In the metaphysis, there was deficient formation of osteoblasts leading to a decrease in the number and size of primary spicules. Vascularization and ossification of the shaft were likewise decreased. The decreased growth processes in the epiphyseal cartilage are comparable to those observed in growing rats kept on pantothenic acid-deficient diet;[2] they are compatible with and account for the reduced growth rate of mice thus treated.[1] The skeletal changes observed in this series of mice are different from those seen in animals kept on a quantitatively reduced but qualitatively adequate diet. In the latter, growth processes proceed at a decreased rate;[4] ossification may be increased in spite of the decreased growth rate, and the bone marrow undergoes serous atrophy. In pantothenic acid deficiency, ossification ceased with the proliferation and development of the cartilage, and no changes in the bone marrow occurred. In the male, these changes were accentuated after only one week, whereas in the female the growth inhibition reached a similar degree after 2 weeks. After 4 weeks, animals of both sexes showed about the same changes in the growth zones. Since, at this age, the normal growth rate in males is greater than that of the females, it is not surprising that the male responded more readily to the vitamin deficiency than the female. The skeletal tissues of the adult mouse are not as dependent on this vitamin as those of the growing animal.

Summary. In growing mice, pantothenic acid is essential for the growth of the skeleton. Deficiency of this vitamin causes inhibition of growth and endochondral ossification. The male reacts more quickly to lack of pantothenic acid than the female. On feeding an adequate stock diet subsequent to a prolonged deficiency, growth of cartilage and ossification are resumed rapidly and may surpass in intensity the processes seen in normal nondeficient animals. In the adult mouse, skeletal changes were not observed under the influence of pantothenic acid deficiency.

---

[4] Silberberg, M., and Silberberg, R., *Arch. Path.*, 1940, **30**, 675.

15609

Tests for Hepatic Dysfunction of Mice.*

J. CASALS AND PETER K. OLITSKY.

From the Laboratories of The Rockefeller Institute for Medical Research, New York City.

Attempts to transmit infectious hepatitis and homologous serum jaundice to laboratory animals have been made by several investigators with results that are negative or inconclusive. In this laboratory we have tried to infect mice with human material derived either from spontaneous cases of infectious hepatitis or from artificially-induced serum jaundice. These experiments are still in progress.

In the course of the latter work it was thought that mice may develop experimentally an inapparent disease with hepatic damage, without showing objective signs. In such a case it would be advantageous (a) to recognize the condition and (b) to follow the course of the damaged liver without sacrificing the animal to observe the lesions. Also, if blind passages were attempted, the knowledge of the state of the hepatic function might give an indication as to the best time to carry out such passages. For these reasons we undertook an investigation of tests of hepatic dysfunction in Swiss mice,

---

* We thank Dr. H. A. Schneider for his help in the studies here reported on *S. enteritidis* and dietary influences. We are also indebted to Dr. R. E. Shank for his cooperation.

applying some of the methods used in clinical medicine.

Owing to the large numbers of animals used, as well as to the technical difficulties, such as obtaining blood serum, these tests had to be modified. Some of the methods, such as those involving collection of urine or feeding of known quantities of materials, were eliminated as impractical. A selection was made of the remaining tests, chiefly on the basis of their practicability in the mouse. The following were therefore investigated: retention of bromsulphalein; bilirubin in the blood; thymol-turbidity; plasma-coagulation time, and vaginal-smear cytology. Tests based on vaginal-smear cytology, although not used in human beings, were studied because an impairment in the ability of a damaged liver to inactivate estrogens[1] and the occurrence of an increased amount of the latter in the circulation of males with hepatic diseases[2] were recently reported.

For each of these tests the normal value or base line was established in control mice. Hepatic lesions were induced in mice by several means, such as by chemical injury with carbon tetrachloride, phosphorus and allyl formate; by bacterial infection with *Salmonella enteritidis;* by injection of hepatic tissue autolysates, and by starvation. The possible lesions that may be induced through dietary factors are now being investigated. Of all the tests employed retention of bromsulphalein proved to be the most dependable and is therefore reported in detail. The types of hepatic lesions in mice are presented first, followed by the description of the tests and the results obtained with them.

Hepatic Lesions in Mice. Carbon Tetrachloride. The toxic effect of carbon tetrachloride on the liver is well known. We followed the method of Cameron and Karunaratne,[3] who studied its toxicity in rats, although the mice were given larger amounts of the chemical per unit of body weight. Mice were injected subcutaneously with a mixture of 9 parts of carbon tetrachloride and 1 part of mineral oil. The amounts varied from 0.2 to 1 cc which represented from 1 to 5 cc of carbon tetrachloride per 100 g of body weight. Immediately after injection, mice became ill with cyanosis and dyspnea, a condition lasting from 10 to 30 minutes. With the larger doses of the chemical (0.5 to 1 cc), all mice appeared sick 24 hours later and about 25% of them died; within the next 4 or 5 days half the number succumbed. The survivors gradually improved and within one week after injection were completely recovered. With the smaller doses (0.2 or 0.3 cc), the symptoms were less pronounced and about 20% died.

Histological examinations of the liver 1, 2, 4, 6, 10, 15 and 27 days after the injection of carbon tetrachloride revealed lesions comparable to those described by Cameron and Karunaratne[3] in rats. At 24 hours there was a considerable degree of necrosis located about the central vein, involving at times most of the hepatic section. Periportal hepatic cells were not visibly damaged. This picture remained for another day or 2 after which new growth of hepatic cells occurred, as indicated by the presence of mitotic figures, along with progressive diminution of necrotic areas. At about the 6th day there was a great deal of diffuse cellular infiltration (polynuclear and mononuclear cells), necrotic areas were greatly reduced in size, and restoration of the architecture of the hepatic-cell cords was in progress. By the 10th day the appearance was practically normal.

Phosphorus. The administration of phosphorus to mice in order to induce hepatic lesions was not found practical. Phosphorus was given either by mouth or by subcutaneous injection, as a commercial rat-poison preparation containing yellow phosphorus. Neither route was successful. Mice refused as a rule to eat phosphorus-contaminated food, and when injected subcutaneously, phosphorus caused the development of local lesions with suppuration and the substance probably failed to reach the liver. As a consequence, only a limited number of mice were affected.

---

[1] György, P., PROC. SOC. EXP. BIOL. AND MED., 1945, **60**, 344.

[2] Gilder, H., and Hoagland, C. L., PROC. SOC. EXP. BIOL. AND MED., 1946, **61**, 62.

[3] Cameron, G. R., and Karunaratne, W. A. E., *J. Path. and Bact.*, 1936, **42**, 1.

Histological examination revealed in these cases extensive hepatic necrosis with marked fatty infiltration and occasional small hemorrhage.

Allyl Formate. When injected into rats, allyl formate brings about typical hepatic lesions consisting of congestion of the sinusoids and hemorrhages which are visible within 1 or 2 hours after injection. Later, about 6 to 8 hours, there is periportal necrosis which is more pronounced in those animals that survive for 24 hours.[4] Mice were injected with allyl formate intraperitoneally, 0.0015 g dissolved in saline per 100 g of body weight. Of 21 mice, 4 died within 3 hours, 10 more died within 24 hours and the remaining 7 were killed or died between 24 and 30 hours. The histological picture was the same as described in rats, namely, sinusoidal congestion and hemorrhages at 3 hours, followed by vast periportal necrosis, visible to the naked eye, after 24 hours.

Salmonella enteritidis. Mice given by mouth a culture of *S. enteritidis* of low virulence, usually exhibited a mortality rate of about 20%, and did not regularly develop lesions in the liver. Mice injected intraperitoneally with a virulent strain, which in dilution of 10^{-6} was uniformly lethal in 5 or 6 days, showed in all instances mild lesions consisting of a slight congestion of sinusoids and edema within 2 or 3 days. Three or 4 days later, when sick or prostrate, these mice had advanced hepatic damage with focal necrosis, polynuclear and mononuclear cell infiltration both diffuse and in abscess-like collections, and marked edema.

Mouse-liver Autolysate. Injection into animals of extracts of organs derived from homologous species induce definite lesions.[5-7] By injecting mice with normal mouse-liver autolysate it has been possible to induce hepatic dysfunction and lesions. A group of 40 mice was injected as follows: a 10% normal mouse-liver suspension in saline was made up and merthiolate in final concentration of 1:10,000 was added. The suspension was kept at 37°C for 24 hours, then at room temperature for about 40 days. Beginning on the 5th day, mice were injected intraperitoneally with 1 cc. Similar injections were repeated every 3 or 4 days until a total of 10 injections was given. No mouse became ill or died during the course of the experiment. One month after the last treatment, 10 mice were killed and their livers were examined. Four showed gross changes, consisting of reduction in size of the liver, which had blunt, rounded edges, and a somewhat irregular surface with scar-like retractions. On microscopic examination these livers showed thickening of Glisson's capsule, increased periportal connective tissue, 1 or 2 cysts, focal necrosis and cellular infiltration. Surviving mice are still being observed.

Starvation. When mice were starved, death occurred in some on the 5th day, in most on the 6th and 7th days and one single mouse, of a total of 30, survived at least 8 days. Two mice were sacrificed daily and sections of the liver were made. In the first 3 days of starvation a mild fatty infiltration was noticed. After that some of the livers showed an increased fatty infiltration with cells harboring large fat droplets or, more often, the amount of fat receded and necrotic lesions about the central vessels appeared. The amount of necrosis was increased after 7-8 days; this necrosis affected first only the cytoplasm, later the nuclei.

Tests of Hepatic Dysfunction. In this section will be described the tests of hepatic dysfunction and their results, and the correlation of the latter with the degree of hepatic damage.

Thymol-turbidity Test. The method followed that of Maclagan[8] except that 0.1 instead of 0.05 cc of serum was used. The resulting opalescence was read in a Coleman spectrophotometer at lambda = 650 mμ and was expressed in terms of turbidity units by multiplying the optical density reading by 100. The units thus calculated agreed fairly closely within the range of values encountered with the units obtained from a standard curve derived from known amounts of bovine albumin precipitated with sulfosalicylic acid.

---

[4] Rosin, A., and Doljansky, L., *Am. J. Path.*, 1946, **22**, 317.

[5] Rivers, T. M., Sprunt, D. H., and Berry, G. P., *J. Exp. Med.*, 1933, **58**, 39.

[6] Morgan, I. M., *J. Bact.*, 1946, **51**, 53.

[7] Oettel, H., *Z. klin. Med.*, 1942, **141**, 773.

[8] Maclagan, N. F., *Brit. J. Exp. Path.*, 1944, **25**, 234.

For this, as well as for the other tests, mice were bled from the heart under ether anesthesia. Only sera showing no or slight hemolysis were used. In general, mice were bled after withholding food overnight, since fed mice gave somewhat higher values, due probably to the presence of fat in the serum. Normal mice, not older than 8 months, and mice injected with a variety of materials such as 1 or 2 injections of mouse-liver suspensions, human serum or feces, gave readings ranging from 0.5 to 4 units. Of 275 sera from 150 mice, only 4 showed readings of 4 or 5 units. The over-all average for these sera was 1.7 units, and the averages for 25 separate groups varied from 1.5 to 2.6 units each.

Normal untreated mice, 14 and 18 months old, exhibited decidedly higher values; 16 such mice had an average of 5.5, and 9 of 16 showed 5 units or more (up to 15.1). Sections of the liver from 3 mice in this group revealed certain inclusion-body lesions as already described.[9]

Mice given phosphorus, carbon tetrachloride or liver autolysate, tested at the time when histological changes were found and when bilirubin or bromsulphalein-retention tests were positive in many, rarely exhibited thymol-turbidity higher than 4 units, the highest being 6.1. Of 120 sera tested, only 6 were higher than 4, with an over-all average of 2.1 units.

Mice inoculated with serum or feces deriving from human cases of infectious hepatitis, and then tested at different intervals after injection, likewise offered no indication of any rise in thymol-turbidity units. Two sera showed 6.9 and 5.2 units; the average value of 107 sera being 2.1 units.

Thus, the thymol-turbidity test in mice reveals that with 0.1 cc serum, an average of 1.7 units was obtained in normals or in animals injected with normal human sera or feces, or mouse-liver extract. Most of the individual readings were between 1 and 3 units.

In view of the results obtained with carbon tetrachloride, phosphorus, or human material derived from infectious hepatitis cases, the thymol turbidity test appeared to be valueless for the detection of hepatic changes induced in mice by these materials.

Plasma-coagulation Time. The coagulation time, as determined by the method of Howell, of recalcified plasma from normal mice was found to be from 1.5 to 4 minutes. Recalcified plasma from mice with hepatic lesions induced by carbon tetrachloride had a similar coagulation time.

Vaginal-smear Cytology. Studies of the estrous cycle were made, observing the cytology of vaginal smears in mice,[10] both normal and those with lesions caused by carbon tetrachloride. It was expected that if estrogen were kept in the blood at a continued high level as a consequence of damage produced in the liver by carbon tetrachloride, it might result in a prolongation of the estrus stage, detectable by the type of cells in the vaginal secretions. Daily observations for 10 to 15 days failed to show any difference between mice given carbon tetrachloride and controls. The estrous cycle was not affected, therefore, by extensive hepatic damage caused by poisoning with carbon tetrachloride.

Cephalin-cholesterol Flocculation Test. This test as described by Hanger,[11] when applied to normal mouse sera gave positive reactions in all cases. The degree of reaction was the same as that shown by mice with extensive hepatic lesions; therefore, it is not useful for the detection of such damage. Positive cephalin-cholesterol flocculation by normal sera has been previously described in several other animal species.[12]

---

[9] Olitsky, P. K., and Casals, J., PROC. SOC. EXP. BIOL. AND MED., 1945, **60**, 48.

[10] Snell, G. D., editor, *Biology of the Laboratory Mouse,* by the Staff of the Roscoe B. Jackson Memorial Laboratory, Blakiston Company, Philadelphia, 1941, pp. 65-71.

[11] Hanger, F. M., *Tr. Assn. Am. Physn.*, 1938, **53**, 148.

[12] Recant, L., Chargaff, E., and Hanger, F. M., PROC. SOC. EXP. BIOL. AND MED., 1945, **60**, 245.

Quantitative Determination of Blood-bilirubin. The method of Evelyn and Malloy, conveniently modified, was used for determination of bilirubin. Sera (or plasma) from mice kept off feed overnight was diluted 6 or 8 times in distilled water. The reagents were added to the test tubes as follows: methyl alcohol, 1 cc; diazo reagent (or diazo blank), 0.2 cc; serum in dilution 1:6 (or 1:8), 0.8 cc. After standing for 30 minutes at room temperature, the color reaction was read in the Coleman spectrophotometer at lambda = 550 mμ. From the standard curve obtained with known amounts of bilirubin, the bilirubin content in mouse serum, in mg per 100 cc, was determined by multiplying the amount given by the meter reading by the dilution factor, 6 or 8.

In 66 normal mice the average amount of bilirubin in 100 cc of serum (or plasma) was 0.3 mg; most values (55 out of 66) were lower than 0.5 mg; the highest was 1.2 mg.

In 40 mice given phosphorus, subcutaneously or orally, the average amount of bilirubin was 0.4 mg per 100 cc of serum, with similar distribution to that of the controls, *i.e.,* 28 of 40 were lower than 0.5 mg. As described in the previous section, most of these mice failed to be affected by phosphorus. However, even in 6 or 8 mice, which were visibly ill and had hepatic lesions, the bilirubin in plasma was not high; the highest in these was 1.2 mg per 100 cc of plasma, and the average was about 0.6 mg.

In 33 mice injected with carbon tetrachloride and tested within 7 days, the average bilirubin in 100 cc of plasma was 0.6 mg; 24 of the 33 had 0.5 mg or higher. The highest reading encountered was 1.6 mg. When mice injected with carbon tetrachloride were tested after 8 to 26 days, the average in 12 mice was 0.3 mg per 100 cc, with 10 lower than 0.5 mg, the highest value was 0.7 mg.

These observations show that the amount of bilirubin in the plasma of mice with hepatic lesions induced by phosphorus or carbon tetrachloride, is somewhat higher than in control mice; however, this increase is neither constant nor pronounced.

Bromsulphalein-retention Test. Of the tests investigated, retention of bromsulphalein has been the most sensitive and accurate indicator of the lesions brought about in the liver by the means already described. In a few instances lesions existed without influencing the retention of bromsulphalein and not always did the degree of retention reflect the extent of the hepatic damage. In general, however, in mice both in groups or as individuals, bromsulphalein-retention was found to be a reliable test for detecting hepatic damage.

In order to develop a method applicable to mice a number of modifications were tried, involving the route of injection of the dye (intravenous, intraperitoneal, subcutaneous), amounts of dye and time of bleeding. A method was finally evolved which is practical and simple even for large numbers of mice. It yielded the largest number of positive results in the presence of hepatic lesions, was practically free from false positives, and within limits, permitted the testing of mice repeatedly with a minimum of deaths.

Method. Mice are weighed individually to the nearest gram. Bromsulphalein is injected intraperitoneally, 0.05 mg in 0.05 cc physiological saline per g of body weight. Thirty minutes later the mouse is bled from the heart; 0.5 cc of blood is deposited in a 6 mm diameter tube containing 0.05 cc of a 2% solution of potassium or sodium oxalate in saline. The blood is centrifuged at 1500 r.p.m. for 15 minutes and the plasma is removed with a capillary pipette. As a rule, 0.22 to 0.25 cc of plasma is obtained; 0.1 cc of plasma is placed in each of 2 Coleman cuvettes (N° 6-308C) and 1.5 cc of physiological saline is added to each, a dilution of 1:16 of the plasma. Then, to the first cuvette is added 0.05 cc of a 10% solution of sodium hydroxide, to the second 0.05 cc of a 10% dilution of hydrochloric acid. A control, consisting of a known amount of bromsulphalein in saline solution is included in each test. The colorimetric reaction is read in the spectrophotometer at lambda = 580 mμ. The values for light transmission are compared with those shown in the standard curve obtained with known amounts of

TABLE I.
Bromsulphalein-retention in Mice Injected with Carbon Tetrachloride.

Days after injection	No. of tests	Bromsulphalein in plasma in mg per 100 cc			% of individual-values higher than 1 mg
		Avg	Highest	Lowest	
1	27	8.4	27.2	2.3	100
2	22	10.6	21.4	2.1	100
3	5	4.1	10.9	.3	60
4	23	2.6	10.1	.4	83
6	6	2.4	6.2	.4	50
8	16	.4	1.1	.2	6
9	11	.9	1.7	.3	27
11	10	.3	.45	.2	0
16	12	.3	.45	.1	0
27	7	.6	1.0	.3	14
31	7	.4	.6	.3	0
Controls, no carbon tetrachloride	346	.46	2.0	.1	4.6

bromsulphalein, read in mg per 100 cc of plasma. Since the plasma is diluted 16 times one must multiply this amount by 16, thus obtaining the amount of bromsulphalein in undiluted plasma. By staggering the injection of dye and bleeding, it is possible to test 20 to 30 mice in about 2 hours. The result of the test is not expressed as the percentage of bromsulphalein-retention as is done in human tests, but rather as the amount of bromsulphalein in plasma, in mg per 100 cc. It should be stated here that in this test (as well as in the determination of bilirubin) the amount of dye in mg per 100 cc of plasma must be considered only as an approximation, hence these tests are not to be taken as exact biochemical determinations. Furthermore, the readings are made in a region where the instrumental error is highest, and the necessity of diluting the plasma introduces still another source of error. However, within these limitations the test has proved workable and dependable in mice and since it has been more sensitive than the other tests here described, it was used extensively. The intraperitoneal injection of dye was used in preference to the intravenous because the former is more practical and is equally sensitive: the absorption of bromsulphalein from the peritoneal cavity began immediately and lasted from 2 to 18 hours. This was determined by titrating the amount of dye left in the peritoneal cavity washed with saline. The elimination of dye began in less than 5 minutes, after injection, as was shown by its recovery from the intestinal contents. A balance is probably struck between the rate of absorption from the peritoneal cavity and the rate of elimination by the liver, which results in a shifting level of bromsulphalein in the blood. There have been no visible ill effects resulting from the injection of this relatively large amount of dye in mice. Moreover, when the same mice were tested 2 or 3 times in succession at 5- or 6-day intervals, there was no indication of an increased retention in the 2nd or 3rd test due to cumulative effects.

TABLE II.
Bromsulphalein-retention in Mice Given *Salmonella enteritidis* of Low Virulence.

Days after injection	No. of tests	Bromsulphalein in plasma in mg per 100 cc			% of individual values higher than 1 mg
		Avg	Highest	Lowest	
4	18	.55	1.2	.1	5.5
9	23	.67	2.0	.1	17.4
14	12	1.0	1.8	.3	41.7
21	6	.9	1.7	.4	33.0

TABLE III.
Bromsulphalein-retention in Mice Given *Salmonella enteritidis* of High Virulence.

				Modified methods			
				Bromsulphalein in plasma in mg per 100 cc			
Method of dye test	Treatment	When tested	No. of mice	Avg	Highest	Lowest	% of individual values higher than 2 mg
0.1 mg subcutaneously; bled at 40 min.	Injected	3rd day	8	1.3	3.1	.5	12.0
	Controls		16	1.0	2.3	.4	6.0
0.1 mg intraperitoneally bled at 30 min.	Injected	4th-5th day	14	3.65	5.8	1.7	78.5
	Controls		15	1.75	3.7	.7	26.5

TABLE IV.
Bromsulphalein-retention in Starved Mice.

		Bromsulphalein in plasma in mg per 100 cc			
Days after starvation began	No. of mice	Avg	Highest	Lowest	% of individual values higher than 1 mg
None (control)	24	.53	1.0	.1	4.0
1	26	.88	2.4	.3	26.9
2	26	.70	2.2	.1	26.9
3	24	.90	2.4	.1	45.8
4	20	1.18	2.3	.3	60.0
5	10	1.74	2.5	.2	80.0
6	4	4.2	8.3	2.0	100.0
7	3	2.4	2.6	2.2	100.0
8	1		8.4		100.0

Results. In 138 normal mice the average amount of bromsulphalein in plasma at the end of 30 minutes was 0.47 mg per 100 cc; in 208 mice injected with human serum or feces, both from healthy individuals and from individuals with infectious hepatitis, or with fresh mouse-tissue suspensions, the amount of bromsulphalein averaged 0.45 mg per 100 cc of plasma. Since the results of the tests in these 2 groups were similar, they were pooled. Thus, there were a total of 346 mice with an average retention of 0.46 mg per 100 cc; 83% were lower than 0.7 mg; 63% were lower than 0.5 mg; 4.6% showed readings higher than 1 mg; the highest single value was 2 mg (Table I).

It is evident, therefore, that any individual value of bromsulphalein-retention lower than 1 mg per 100 cc is normal, between 1 and 2 mg per 100 cc may be considered as questionably, and higher than 2 mg as definitely increased.

Of 146 tests with mice injected with carbon tetrachloride (Table I) most of them exhibited within 6 days significantly increased bromsulphalein-retention. As stated in the previous section, this was the time in which lesions were found; after the 8th day neither positive dye-retention nor visible lesions occurred.

Of 14 mice given phosphorus either orally or parenterally, tested on the 5th day, there were 4 values higher than 1 mg of bromsulphalein per 100 cc of plasma, namely, 19.7, 5.7, 1.7 and 1.2. Allyl formate poisoning resulted in retention of bromsulphalein greater than that shown by controls. Of 8 mice tested 3 hours after injection, 3 had values higher than 2 mg per 100 cc and 4 between 1 and 2 mg; the lowest, 0.9 mg and the highest 8.6 mg, with an average of 2.6 mg per 100 cc. Mice receiving liver-autolysate exhibited, after the early injections, an average of 0.35 mg; after all the injections, 0.75 mg. It will be noted that significant differences occur between the bromsulphalein-retention of mice given *S.*

enteritidis and normal ones (Tables II and III).

Finally, mice starved from 1 to 8 days were tested for retention of the dye (Table IV). It is of interest that a mild increase in the retention of bromsulphalein arose even in the early days of starvation. Beginning with the 5th day, however, at a time when either marked fatty infiltration or necrotic lesions was observed histologically and mice had begun to die, the retention of bromsulphalein was decidedly increased, as shown by the fact that all but 2 of the mice tested on the 5th day or later had readings higher than 1 mg.

Summary. Six tests for the study of hepatic dysfunction and damage were applied to Swiss mice. Insufficiently sensitive to detect hepatic lesions were the thymol-turbidity test, plasma-coagulation time and vaginal-smear cytology. Cephalin-cholesterol flocculation was not applicable owing to the fact that normal mouse sera reacted positively. Blood-bilirubin determinations indicated hepatic lesions induced by carbon tetrachloride or phosphorus. The increase in bilirubin in the blood, however, even in cases of extensive damage, was not high; 0.3 mg per 100 cc of plasma in control mice as an average, as compared with 0.6 mg per 100 cc in those with carbon tetrachloride lesions.

Retention of bromsulphalein was the most useful and accurate of all the tests described herein. A base line was established for control mice; *i.e.,* less than 1 mg of dye retained per 100 cc of plasma could be regarded as normal. In mice with hepatic lesions, tested at a proper time and within the limitations mentioned, values 10 or 20 times higher than the normal were not unusual. Since retention of this dye was increased by diverse methods such as chemical poisoning, bacterial infection, starvation, and injection of autolyzed tissue, its use may be of value in the study of experimental problems connected with hepatic injury of mice.

15610

Permanent Ablation of Bone Marrow in Long Bones of Living Rabbits.

BERNHARD STEINBERG AND RUTH A. MARTIN.

From the Toledo Hospital Institute of Medical Research, Toledo, Ohio.

In a previous paper a method of removing marrow from a single bone in living rabbits was described.[1] In this article a procedure is outlined in which the marrow of all the long bones was removed and replaced by inert material. The regeneration of marrow was prevented permanently in the bones so treated. On the basis of Dietz's analyses of distribution of bone marrow,[2] it was estimated that approximately 41 ± 5% of the total marrow may be ablated by this method.

The rabbit was the animal of choice. The marrow in this animal is tubular and with very few connective tissue bands which bind it to the endosteum. The complete marrow is therefore readily expressed. The animal is also sufficiently large to withstand several operative procedures. The marrow was removed from 2 to 10 bones: both tibiae, both femora, both humeri, and both radii and ulnae. The marrow of all other bones is inaccessible by this method. Thirty rabbits were used in the experiment over a period of 3 years.

The animals were anesthetized with pentobarbital sodium. The initial steps in drilling holes in the bone with a Ralk nail drill and expressing the marrow were similar to those already described.[1] Cotton strips of varying lengths and thickness were saturated in bone wax and autoclaved in Petri dishes. The strips of cotton were introduced into the marrow cavity with a thin bladed thumb forceps and forced down with a blunt, fairly flexible probe. Both epiphyseal ends were plugged first with soft bone wax which was followed with wax-saturated cotton. Pressure by the harder cotton occasionally produced shock and death of the animals. The entire marrow cavity was packed tightly. This method of packing resulted in the expulsion of remaining bits of marrow and prevented regeneration of marrow in loosely filled places. Many other procedures in filling the marrow cavity were tried such as filling with a paste by means of needle and syringe. The method described, although more time consuming and laborious, was found to conform to the requirements of the experiment.

---

[1] Steinberg, B., and Martin, R. A., PROC. SOC. EXP. BIOL. AND MED., 1946, **61**, 428.

[2] Dietz, A., PROC. SOC. EXP. BIOL. AND MED., 1944, **57**, 60.

In 24 animals, the marrow of 1 to 3 bones was replaced with inert material at a single operation at intervals of 2 to 4 weeks. In 6 animals, the marrow of all the long bones on one side of the body, tibia, femur, humerus, radius and ulna, was replaced at one operation. The marrow of all the long bones of the other side was removed from 5 to 7 days later. These varying time intervals allowed to study the effects of rapid and gradual ablation of bone marrow.

The marrow cavities and the bones were examined at intervals over a period of 3 years. There was an initial thickening of the bone in the first several weeks followed by a slight degree of reduction in thickness. Whenever the packing was not complete, proliferating endosteum and marrow were found formed between bone and packing.

Summary. A method of ablating permanently the marrow of all the long bones in living rabbits is presented. The ablation may be carried out rapidly over a period of a week or gradually over several weeks. Approximately 41 ± 5% of the total marrow of the animal may be ablated permanently by this method.

15611

Effects of Permanent Mechanical Ablation of Marrow of Long Bones on Peripheral Blood of Living Rabbits.

Bernhard Steinberg and Ruth A. Martin.

From the Toledo Hospital Institute of Medical Research, Toledo, Ohio.

We described in previous articles procedures of removing marrow from long bones of living rabbits and replacing it with an inert material.[1,2] The total marrow of the body may be reduced by 41 ± 5% by this method.[3] Thirty rabbits were used over a period of 3 years in this experiment. In 24 animals the marrow was removed gradually over a period of 22 days to 5 months. In 6 rabbits the marrow was replaced within one week. The 30 animals were observed for periods of 10 days to 14 months after ablation of the marrow. Marrow was removed from 2 to 10 of the long bones with a reduction of 11 to 41% of the marrow. The marrow of the radius and/or ulna on one side was left frequently intact. The marrow of these bones is completely inactive in mature animals. Peripheral erythrocyte, platelet and leukocyte counts, hemoglobin determinations and studies of stained blood smears were made at frequent intervals before and after removal of marrow.

After removal of marrow there was an initial rise in the number of peripheral leukocytes in most of the animals. In some of the rabbits there was a drop. The change in the peripheral content of the cells re-

---

[1] Steinberg, B., and Martin, R. A., Proc. Soc. Exp. Biol. and Med., 1946, **61**, 428.

[2] Steinberg, B., and Martin, R. A., Proc. Soc. Exp. Biol. and Med., 1946, **63**, ...

[3] Dietz, A., Proc. Soc. Exp. Biol. and Med., 1944, **57**, 60.

TABLE I.
Effect of Rapid Removal of 41 ± 5% of Marrow from Long Bones on Cell Content of Peripheral Blood.

Time examined	Hemoglobin, in g	Erythrocytes, millions	Leucocytes, thousands	Platelets, thousands	Differential study of stained blood smears		
					Granulocytes	Lymphocytes	Monocytes
Before removal of marrow	11.5 to 12.5 in 1 wk	5.3 to 6.0	8,200 to 9,200	330,000 to 350,000	34	62	4
One day after removal	9	4.8	7,100	210,000	34	64	2
In 3 days	8.7	4.4	19,600	280,000	31	68	1
,, 5 ,,	8	4.5	20,800	300,000	22	76	2
,, 7 ,,	10	5.0	12,200	270,000	22	76	2
,, 20 ,,	10.5	5.0	9,800	320,000	41	56	3

TABLE II.
Infrequent Effects of Gradual Removal of Marrow from Long Bones on Cellular Content of Peripheral Blood.

Time examined	Hemoglobin, g	Erythrocytes, millions	Leukocytes, thousands
Before removal of marrow	12.8	5.2	12,250
After removal of 1 marrow, 7 da. later	13.9	6.0	15,200
3 marrows, 14 da. later	12.5	5.2	5,550
4 marrows, 14 to 19 da. later	12.0	5.1	16,900 to 25,850
7 marrows,* 5 da. later	13.0	6.3	49,800
Before removal of marrow	9.5	4.5	13,000 46 polys. 54 lymphs
9 da. after removal of 2 marrows†	7.3	2.8	135,300 52 segmented polys. 11 stab polys. 13 juveniles 14 myelocytes 10 lymphs

* Animal was ill. It was killed for study of tissues. Platelets were slightly reduced for a week after removal of 3 marrows, but returned to normal. The differential cell content was not significantly altered.

† Animal died on tenth day. Platelets not significantly affected.

mained for 4 to 7 days and was reflected by a rise or drop either in granulocytes alone or in lymphocytes and granulocytes. The erythrocytes and the hemoglobin either remained unchanged or were slightly decreased in some animals. The leukocytes, erythrocytes and the hemoglobin content eventually returned to normal, but in a few instances remained slightly decreased. The platelets were at first reduced in number, but in most animals returned to a normal level (Table I).

When more than 6 marrows were ablated, some of the animals developed a permanent slight reduction in leukocytes, erythrocytes, platelets and hemoglobin. In 7 out of 30 rabbits there occurred significant changes in the peripheral cell content. The leukocytes were increased appreciably, reaching from 20,000 to 135,300 mm^3 of blood. The rise was contributed by granulocytes. In 2 animals there were immature granulocytes, which constituted from 17 to 27% of the total number of cells. The hematologic picture was that of leukemia. The animals either became very ill and were killed or they died. The erythrocytes and hemoglobin did not reveal changes in those rabbits with an increase in mature granulocytes. However, immaturity of granulocytes appeared to be associated with a decrease in hemoglobin and erythrocytes and with presence of normoblasts and poikilocytes (Table II).

Summary. Marrow was removed from long bones of rabbits and replaced by inert material. This procedure resulted in a permanent suppression of marrow. From 11 to 41% of the total marrow was obliterated by this method. The effects on cell and hemoglobin content of peripheral blood were investigated. In most of the animals there were no significant permanent changes. Extirpation of the marrow was followed frequently by an increase in leukocytes and a decrease in hemoglobin, erythrocytes and platelets. When more than 6 marrows were ablated, some of the animals developed a slight reduction in all the elements investigated. An infrequent animal showed significant changes. Leukocytes were increased in number and the peripheral blood was suggestive of leukemia. Appearance of immature granulocytes and a high leukocyte count was associated with a reduction of hemoglobin and erythrocytes and morphologic abnormalities of red blood cells.

15612

Tissue Changes in Rabbits with Ablation of Marrow of Long Bones.

Bernhard Steinberg.

From the Toledo Hospital Institute of Medical Research, Toledo, Ohio.

In previous articles a method of replacing marrow of long bones with inert material was described.[1,2] The procedure reduces the marrow content of the body of the rabbit by approximately 41 ± 5%.[3] In this paper tissue changes incident to the substitution of marrow by inert material are described. Thirty rabbits were used in this experiment over a period of 3 years. Some of the animals died and others were killed in from 10 days to 14 months after removal of the marrow. In the 30 rabbits, 11 to 41% of the marrow was ablated in from 4 days to 5 months. Gross and histologic observations were made of all the thoracic and abdominal viscera, the remaining marrow and occasionally of the pituitary gland.

In 6 animals 41 ± 5% of the total marrow was replaced by inert material over a period of 2 to 5 months and the animals were allowed to survive for total periods of 4 to 14 months. Of the 6 animals, 4 showed infiltration by mature and immature granulocytic leukocytes of liver, kidneys, spleen and in 2 instances of the heart muscle and lungs. One animal showed a slight degree of infiltration of liver and kidneys and in the sixth there were no infiltrative changes (Fig. 1).

In 14 animals, 37 ± 5% of the marrow was ablated. The animals were allowed to survive from 2 to 14 months. Nine of the rabbits developed cell infiltration of liver, kidneys, spleen and in some there was involvement of the adrenals. Four rabbits showed a slight infiltration of liver and spleen only. One animal did not have any infiltrative changes.

In 7 rabbits 29 to 37% of the marrow was replaced by inert material. The total survival period of each animal did not exceed 2 months. Very slight cell infiltration was found in one animal. There were no infiltrative changes in the others.

In 3 animals from 11.5 to 23% of the bone marrow was removed and the total survival period varied from 1 to 4 months. There was no cell infiltration in any of the organs (Table I).

The unextirpated marrow from ribs, ulna and radius was correlated with the infiltrative changes in the other organs. The radius and ulna in an adult rabbit are acellular and were used as a criterion for the demand

---

[1] Steinberg, B., and Martin, R. A., Proc. Soc. Exp. Biol. and Med., 1946, **61**, 428.

[2] Steinberg, B., and Martin, R. A., Proc. Soc. Exp. Biol. and Med., 1946, **63**, ...

[3] Dietz, A., Proc. Soc. Exp. Biol. and Med., 1944, **57**, 60.

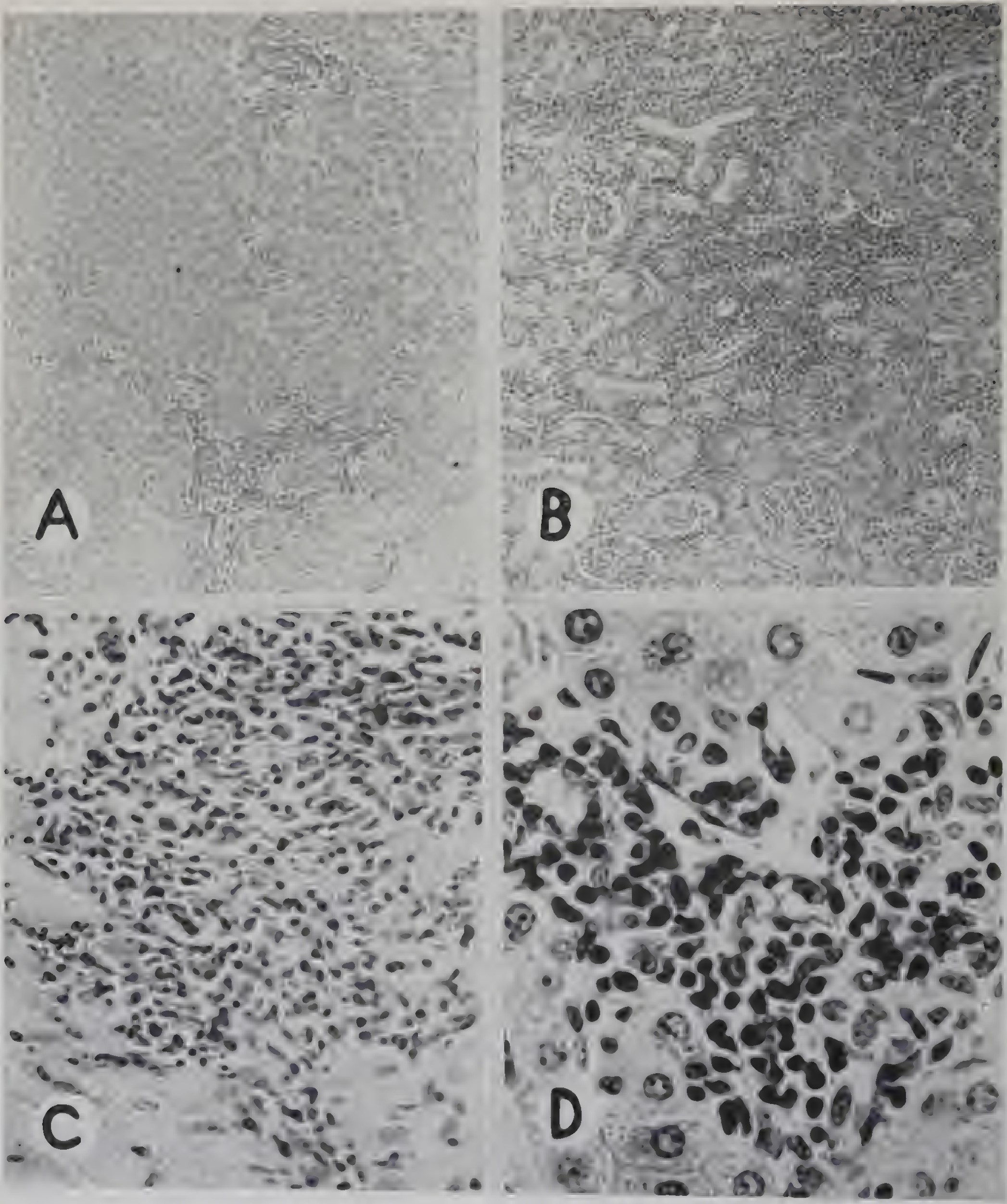


Fig. 1.

Infiltration of organs by mature and immature granulocytes after extirpation of marrow of long bones. A. Liver with patchy cell infiltration. B. Kidney with a patch of cell infiltration. C. Heart muscle with patchy cell infiltration. D. Higher magnification of cell infiltrate of liver.

of cells by the organism. The rib marrow is normally cellular with a fairly constant proportion of cell types. This marrow served as indicator of change in cell type. Cellular infiltration of the viscera, usually, coincided with the degree of cell hyperplasia in the remaining marrow. However, in some of the rabbits hyperplasia of the marrow was not

TABLE I.
Effect on Tissue After Permanent Ablation of Bone Marrow in Living Rabbits.

No. of rabbits	% of marrow replaced by inert material	Total duration of exper.	No. of animals with degree of cellular infiltration of organs	Organs involved by cell infiltration	Cellularity of usually inactive marrow	Size of adrenals in mg	Max. peripheral leukocyte count per cu mm of blood
6	41 ± 5	2 to 14 mo.	2 +++	liver, kidneys, spleen, heart and lung.	3 very cellular 2 slightly 1 acellular	400 750 850 950 1070 1075	10,800 to 19,600
			2 ++	liver, kidneys and spleen.			
			1 ±	kidneys and spleen.			
			1 neg.				
14	37 ± 5	2 to 14 mo.	3 +++	liver, kidneys, heart, spleen and lymph nodes	9 very cellular 4 slightly 1 acellular	2-300 or less 4-860 or less 8-1850 or less	12,300 to 49,800
			6 ++	liver, kidneys and spleen			
			4 +	liver and either spleen or lymph nodes.			
			1 neg.				
7	29 to 37 ± 5	10 da. to 2 mo.	1 +	liver, lungs and spleen.	very cellular with increase of immature forms	850 to 1075	16,000 to 135,300
			6 neg.				
3	11.5	2 mo.	”	neg.	moderately to very cellular	275	18,000
	16	1 ”	”	”		315	17,950
	23	4 ”	”	”		350	30,800

neg. No cell infiltration.
± Questionable infiltration.
+ Very slight infiltration.
++ Slight to moderate infiltration.
+++ Moderate infiltration involving more than 2 organs.

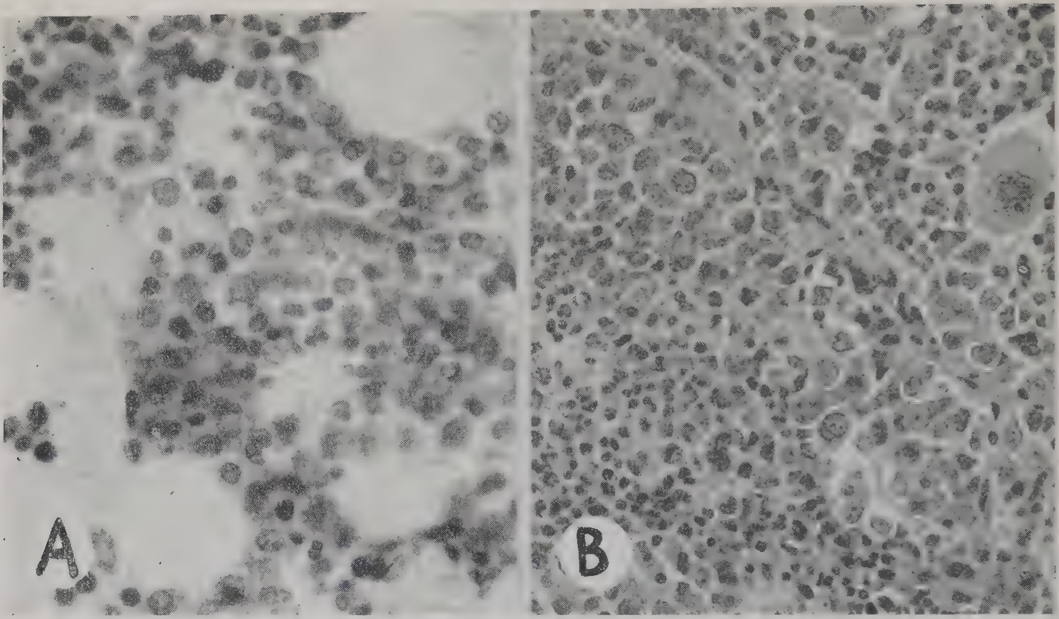


FIG. 2.

Effect of replacement of marrow of long bones with inert material on remaining marrow. A. Marrow of radius. In normal adult rabbits this marrow is aplastic. In this instance the marrow is hyperplastic and contains mostly immature cells. B. Rib marrow. There is considerable increase in cellularity with a change to a predominantly immature type of cell.

associated with infiltration in other organs. In 2 instances with organ infiltration, the marrow of radius and ulna was aplastic and only moderately cellular in the ribs. (Fig. 2).

The adrenal gland in most instances of marrow extirpation became increased in size. The increase was due to hyperplasia of the cortical cells and largely of the zona fasciculata. The average weight of both adrenals in 25 normal rabbits was 260 mg. Observation of adrenal weight in many other normal rabbits disclosed an occasional large pair adrenals with maximum weights of 700 to 1275 mg. Neither the age nor the weight of the animal affected significantly the weight of the adrenals. In animals with more than 16% extirpated marrow the average weight of adrenals was 867 mg. In many individual animals the weight of the glands exceeded 1000 mg. (Table I).

Summary. From 11.5 to 41 ± 5% of the total bone marrow was extirpated in 30 rabbits and the bone cavities were filled with inert material. The animals were allowed to survive for periods of 10 days to 14 months. Tissue changes were correlated with the quantity of marrow removed and the duration of survival of the animal. In some of the organs there appeared a cell infiltration of mature and immature granulocytes similar to that seen in human aleukemic leukemia. The marrow of the radius and ulna, normally aplastic for an adult rabbit, and the rib marrow showed marked cellularity in most animals. There was a considerable number of immature granulocytes in the marrow of some rabbits. The adrenal glands were increased in weight due to hyperplasia of the zona fasciculata.

15613 P

Carotid Receptors Essential in the Gasping of the Isolated Rat Head.

R. D. Tschirgi. (Introduced by R. W. Gerard.)

From the Department of Physiology, University of Chicago.

Selle, Hiestand and others[1-4] have demonstrated gasping phenomena in the isolated ischemic rat head and determined the variations with age and drug administration prior to decapitation. In duplicating these experiments a single edge razor blade held vertically in a blade holder was used,[4] and the section made from the dorsal surface as far cephalad as possible. Subsequent examination of the heads revealed that the section had passed caudal to the carotid sinus-body region leaving this mechanism intact. To evaluate the possible role of this structure in the gasping phenomena, male rats were anesthetized with chloralose (0.4 g per kg body weight), previously shown to increase the survival time and total number of gasps more than 250% in rats of a younger age group,[1] and the carotid sinus body located bilaterally through a midline incision. Decapitation was then performed in one group of 10 animals caudal to these structures and in another group of 10 above them, the surgical technic being as identical as possible in all cases. The rats weighed from 50 g to 64 g, a range previously shown to have a characteristic and fairly constant gasping response. The results shown in Table I indicate that only when the carotid mechanism remains connected to the brain does gasping occur.

Since rats of this weight group show both an initial aerobic series of gasps and a later anaerobic series[4] it must be concluded that the carotid drive is essential to both.

TABLE I.
Effect of Removal of Carotid Sinus-body on Gasping in Isolated Rat Head.

	No. of rats	Avg gasping time	Avg No. of gasps
Carotid mechanism intact	10	83 sec	18
Carotid mechanism removed	10	0	0

In 2 dogs (7.6 and 11.3 kg) under nembutal anesthesia gasping (depression of the mandible) was produced by occluding the trachea which was subsequently released and artificial respiration instituted to restore the animals when respiratory efforts had ceased. After removal of the carotid sinus-body region by resecting this area of the common carotid artery and its branches (ext. carotid, int. carotid and occipital artery), tracheal occlusion caused marked diaphragmatic and intercostal hyperpneic efforts, but no mandibular movements occurred. Removal of the carotid reflex mechanism caused, of itself, no change in respiratory rate or depth prior to tracheal occlusion.

On the basis of the above findings, Hiestand and Thoms reinvestigated the absence of gasping in mice. They have shown (private communication) that in this species the carotid body lies at the level of the pectoral girdle and that the section must be caudal to the forelegs before any gasping will occur.

Thus the primary drive to the respiratory center producing mandibular movements in the ischemic rat head, at least under chloralose anesthesia, is a reflex from the carotid receptors. This explains in part why some narcotic drugs decrease survival time and number of gasps in the ischemic head (ether, chloroform, nembutal) whereas others (chloralose) greatly increase these factors. The latter, although diminishing the response of the center to direct chemical influence, increases the sensitivity and effectiveness of the carotid chemoreflex system.[5] Further indication that the essential drive producing gasping phenomena is of chemoreflex origin lies in the nature of these structures. Their extreme ruggedness enables them to activate the central neurones under conditions which render these latter unable to respond to autogenous chemical stimuli. The ability of the carotid body to function during severe anoxia provides the organism with a last line of respiratory defense, Schmidt's ultimum moriens of respiratory control.[6]

---

[1] Selle, W. A., Proc. Soc. Exp. Biol. and Med., 1942, **51**, 50.

[2] Selle, W. A., Proc. Soc. Exp. Biol. and Med., 1945, **58**, 24.

[3] Tschirgi, R. D., and Hiestand, Wm. A., *Anat. Rec.*, 1944, **89**, 543.

[4] Hiestand, Wm. A., Tschirgi, R. D., and Miller, H. R., *Am. J. Physiol.*, 1944, **142**, 153.

[5] Schmidt, C. F., *Anesthesia and Analgesia*, 1940, **19**, 261.

---

[6] Bard, P., *MacLeod's Physiology in Modern Medicine*, C. V. Mosby Co., St. Louis, 1941, p. 593.

I am indebted to Dr. R. W. Gerard of the Department of Physiology, University of Chicago, and D. B. Gordon for assistance in the preparation of this report.

15614

Mutual Depression of Reabsorption and Excretory Maxima in Renal Tubules.

C. RILEY HOUCK. (Introduced by Homer W. Smith.)
With the assistance of Mr. Albert Altchek.
*From the Department of Physiology, New York University College of Medicine, New York, N.Y.**

It is generally understood that the capacity of the renal tubule cells to transport a substance in one direction is not influenced by the simultaneous transport of another substance in the opposite direction. Thus Smith *et al.*[1] report that simultaneous determinations of the maximal tubular reabsorption of glucose (Tm_G) and the maximal tubular excretion of *p*-aminohippuric acid (Tm_{PAH}) yield the same values as separate determinations, and elsewhere Smith[2] illustrates a set of simultaneous measurements of these 2 functions. This is also presumably true for the dog.[3,4] However, when individual experiments are examined, there is, in some instances, evidence of depression of Tm_G by PAH and of Tm_{PAH} by glucose. We have therefore reexamined this question of mutual depression of the transport of glucose and PAH in the dog.

Experimental. Tm_G and Tm_{PAH} were determined in 3 trained, unanesthetized female dogs ranging in body weight from 17 to 24 kg. Tm_G alone was determined during 3 consecutive urine collection periods of approximately 10 minutes each. After a priming dose of PAH, this substance was added to the infusion fluid and Tm_{PAH} and Tm_G were determined simultaneously during 3 additional periods. On another day this procedure was repeated in the same dog but the substances were reversed, *i.e.*, Tm_{PAH} alone was measured first. The creatinine clearance was taken as a measure of the glomerular filtration rate. The dogs were hydrated with 50 cc of water per kg of body weight by stomach tube 45 minutes before U_O. Plasma levels of creatinine, glucose and PAH were obtained by suitable priming injections into the jugular vein 10 minutes before U_O and maintained by constant intravenous infusion into a leg vein by a gravity mercury-drip pressure apparatus. In all experiments the load/Tm ratio for both glucose and PAH was well above 1.5. Urine was collected by an indwelling bladder catheter and blood was withdrawn periodically from the femoral artery through a 19-gauge retention needle.

---

* Present address: Dept. Physiology, University of Tennessee, Memphis.

[1] Smith, H. W., Goldring, W., Chasis, H., Ranges, H. A., and Bradley, S. E., *J. Mt. Sinai Hosp.*, 1943, **10**, 59.

[2] Smith, H. W., *Porter Lectures*, Series 9, 1939, Univ. Extension Division, Univ. of Kansas, Lawrence, Kansas. Revised 1943.

[3] Selkurt, E. E., *Am. J. Physiol.*, 1944, **142**, 182.

[4] Eiler, J. J., Althausen, T. L., and Stockholm, M., *Am. J. Physiol.*, 1944, **140**, 699.

TABLE 1.

Mutual Depression of Renal Tubular Maxima (Tm's) of Glucose and *p*-Aminohippuric Acid (PAH) in the Dog.

Dog	Date of exp.	Glucose Tm* Alone mg/min	Glucose Tm* During PAH infusion mg/min	$\frac{\text{Tm}_G \text{ with PAH}^\dagger}{\text{Tm}_G \text{ alone}}$	$\frac{\text{Avg Tm}_G \text{ with PAH}^\ddagger}{\text{Avg Tm}_G \text{ alone}}$	PAH Tm* Alone mg/min	PAH Tm* During glucose infusion mg/min	$\frac{\text{Tm}_{PAH} \text{ with G}^\dagger}{\text{Tm}_{PAH} \text{ alone}}$	$\frac{\text{Avg Tm}_{PAH} \text{ with G}^\ddagger}{\text{Avg Tm}_{PAH} \text{ alone}}$
R	7/24/46	250	228	0.91		—	13.6	—	
	29	238	219	0.92		—	16.6	—	
	8/ 1	—	211	—		19.8	16.7	0.84	
	5	—	181	—		23.6	17.7	0.75	
	Avg	244	210	0.92	0.86	21.7	16.2	0.80	0.75
	σ	11.8	22.8			2.3	2.1		
	Avg-2σ	220				17.2			
A	7/26/46	224	192	0.86		—	13.8	—	
	30	212	186	0.88		—	13.1	—	
	8/ 2	—	209	—		18.3	15.8	0.86	
	7	—	188	—		16.1	13.9	0.86	
	Avg	218	194	0.87	0.89	17.2	14.2	0.86	0.83
	σ	11.7	16.5			1.4	1.7		
	Avg-2σ	195				14.4			
B	8/23/46	172	171	0.99		—	9.9	—	
	9/12	155	135	0.87		—	12.4	—	
	7/19	—	128	—		16.1	12.7	0.79	
	Avg	164	145	0.93	0.88	16.1	11.7	0.79	0.73
	σ	14.9	20.4			0.83	1.6		
	Avg-2σ	134				14.4			
Avg				0.91	0.88			0.82	0.77

* Each value is an average of 3 consecutive 10-minute urine collection periods.

† Ratio based on Tm's studied on same day.

‡ Ratio based on average of all Tm's studied.

σ = Standard deviation from mean.

Plasma proteins were precipitated by cadmium sulfate and NaOH, and creatinine and PAH were analyzed as described by Smith *et al.*,[5] while glucose was determined by Shannon's modification[6] of the Folin method. All colorimetric determinations were made in duplicate and read on an Evelyn photoelectric colorimeter.

Tm_{PAH} in mg/min. was calculated by subtracting the amount filtered per minute (0.83 × plasma concentration in mg/cc × filtration rate in cc/min.) from the total amount excreted per minute (urine concentration in mg/cc × urine flow in cc/min.) Tm_G in mg/min. was calculated by subtracting the amount excreted per minute (urine concentration in mg/cc × urine flow in cc/min.) from the amount filtered per minute (plasma concentration in mg/cc × filtration rate in cc/min.)

Results. The values for Tm_G and Tm_{PAH} determined alone or simultaneously are given in Table I where each value represents the average of 3 consecutive 10-minute urine collection periods. The ratios:

$$\frac{Tm_G \text{ with PAH}}{Tm_G \text{ alone}} \quad \text{and} \quad \frac{Tm_{PAH} \text{ with glucose}}{Tm_{PAH} \text{ alone}}$$

are also presented when values in both denominator and numerator were determined consecutively on the same day. Along with these ratios are included ratios whose numerator and denominator represent the overall average of all values obtained in each category, *i.e.*, on different days as well as on the same day.

It is evident that the tubular transport mechanisms for glucose and PAH in the dog are not mutually independent, as previously reported, because saturation of the transport mechanism by one substance depresses the transport of the other. The depression is of small magnitude and might be dismissed as not significant. However, some depression occurs consistently in each of the 3 dogs studied, and in 2 out of 3 dogs, the mean of all Tm_G values measured simultaneously with Tm_{PAH} fall outside the limit of 2 σ† from the mean of Tm_G measured alone. Likewise, in 2 out of 3 dogs, the mean of Tm_{PAH} measured during glucose saturation falls outside the limit of 2 σ from the mean of Tm_{PAH} measured alone, and is on the borderline of this limit in the third dog. When all data on each dog are averaged, the percentile decrease in Tm_G during PAH infusion is practically the same in each of the 3 dogs. The percentile decrease in Tm_{PAH} during glucose infusion is greater in 2 dogs than in the third. In every case, the percentile depression of Tm_{PAH} is greater than the percentile depression of Tm_G, indicating a more marked effect of glucose on PAH transport than of PAH on glucose transport. Averaging the data of the 3 dogs, Tm_G is depressed 12%; Tm_{PAH}, 23%; *i.e.*, the effect of glucose on PAH transport is nearly twice as great as the effect of PAH on glucose transport.

Selkurt[3] studied Tm_G alone and simultaneously with Tm_{PAH} in 4 dogs, and reported ratios of Tm_G with PAH/Tm_G alone of 0.92, 0.98, 0.88 and 1.06, respectively, with an overall average of 0.96. Therefore, he concluded that PAH did not influence Tm_G. Eiler, Althausen, and Stockholm[4] have reported data on one dog which indicate a slight mutual depression of Tm_G (11%) and $Tm_{diodrast}$ (18%), although they make no particular mention of it. In another dog, $Tm_{diodrast}$ was decreased 12% by glucose. As mentioned above, Smith *et al.*[1,2] have found no mutual interference of glucose and diodrast transport in normal man, and therefore state that tubular maxima of these substances may be studied simultaneously. However, they do not particularly recommend this procedure, "since it offers opportunities for unanticipated complications" (p. 92). In this regard, Klopp, Young and Taylor[7] report a decrease in Tm_{PAH} with glucose in 3 normal individuals, and an increase in Tm_G with PAH in 4 other individuals.

The mutual depression of Tm_G and Tm_{PAH} obtained in the present study may be due to a deflection of available energy necessary for transport[3] rather than to competition for a specific cellular constituent or "B substance" involved in the transfer of solutes across renal tubule cells.[8] It seems unlikely that the same B substance would be used in the transport of solutes in opposite directions within the cell, when solutes traveling in the same direction may have a different B substance, *e.g.*, glucose and glycine.[9] Assuming that the B substance of PAH is different from that of glucose, the cause of mutual interference must be sought in some portion of the transport mechanism common to each. As supporting evidence, Selkurt[3] has pointed out the fact that both Tm_G and $Tm_{diodrast}$ can be reduced by phlorizin.[2] In this regard, it seems more reasonable to suggest that the energy necessary for combination of either glucose or PAH with its B substance, that energy for the release from this substance at the terminus of transport, or that energy required for transport across the cell is not available in sufficient quantity to satisfy the simultaneous demands of PAH and glucose. In other words, this available energy may be the limiting factor in the capacity of the tubules when they are carrying these solutes in opposite directions simultaneously.

---

[5] Smith, H. W., Finkelstein, N., Aliminosa, L., Crawford, B., and Graber, M., *J. Clin. Invest.*, 1945, **24**, 388.

[6] Shannon, J. A., Farber, S., and Troast, L., *Am. J. Physiol.*, 1941, **133**, 752.

† σ = Standard deviation from the mean.

[7] Klopp, C., Young, N. F., and Taylor, H. C., Jr., *J. Clin. Invest.*, 1945, **24**, 117.

Conclusion. In the dog, the maximum renal tubular reabsorption of glucose (Tm_G) and the maximum tubular excretion of *p*-aminohippuric acid (Tm_{PAH}) are mutually depressed when determined simultaneously. The percentile depression of PAH by glucose is twice as great as the depression of glucose by PAH. This depression is attributed to competition for available energy rather than to competition for a common cellular constituent (B substance) involved in tubular reabsorption and excretion.

---

[8] Shannon, J. A., *Physiol. Rev.*, 1939, **19**, 63.

[9] Pitts, R. F., *Am. J. Physiol.*, 1943, **140**, 156.

15615

Improved Fixation for Histological Demonstration of Glycogen and Comparison with Chemical Determination in Liver.*

HELEN WENDLER DEANE, FRANCES B. NESBETT, AND A. BAIRD HASTINGS.

From the Departments of Anatomy and Biological Chemistry, Harvard Medical School, Boston, Mass.

Previous attempts to demonstrate a quantitative correlation between the amount of glycogen present in liver as determined by chemical procedures and as revealed by histological examination have been unsuccessful.[1,2] It is the purpose of the present paper to present data showing that under certain conditions such a correlation can be demonstrated. The increasing number of histochemical studies of tissues employing chemical technics on the one hand and histological technics on the other makes such a comparative study desirable.

This study was undertaken after it had been observed that glycogen can be preserved uniformly throughout histological blocks by placing pieces of organs in ice-cold fixative, instead of fixing blocks at room temperature as had been done heretofore.

Material and Methods. In order to prepare animals with variable concentrations of liver glycogen, a method originally suggested by Higgins, Berkson and Flock[3] was employed. For 5 days before sacrifice, 20 male

---

* This work was done in part under a grant from the American Cancer Society on the recommendation of the Committee on Growth of the National Research Council.

[1] Grafflin, A. L., Marble, A., and Smith, R. M., *Anat. Rec.*, 1941, **81**, 495.

[2] Eger, W., *Virchow's Arch.*, 1942, **309**, 607.

[3] Higgins, G. M., Berkson, G., and Flock, E., *Am. J. Physiol.*, 1932, **102**, 673; *ibid.*, 1933, **105**, 177.

Wistar rats, 3 months old and weighing on the average 307 g, were fed for a 2-hour period each day. Water was available at all times. The rats were killed by concussion, in groups of 4, at 6, 12, 18, 24, and 48 hours after the fifth and last meal. Pieces of the right and left lateral lobes of the liver were immediately dropped into separate bottles of Rossman's picro-alcohol-formalin fixative[4] chilled to 0°C. They were kept in this medium for 24 hours. Adjacent pieces of the 2 lobes, each weighing approximately 1 g, were immersed in weighed flasks containing 30% aqueous KOH at room temperature for the chemical determination of glycogen.

The flasks containing the samples of liver were reweighed and then immersed in boiling water for 30 minutes. Two aliquots of this alkaline digest were analyzed for glycogen by the Good, Kramer and Somogyi modification of Pflüger's method.[5] This method has been modified in the following respects: the whole procedure is carried out in 15 cc graduated centrifuge tubes; for the precipitation of the glycogen, 95% alcohol is added to give a final concentration of about 60%; after the mixture is heated to coagulate the precipitate, the tubes are chilled to 0°C in an ice bath before centrifugation; the supernatant fluid is then discarded, the precipitate dissolved in a minimum quantity of water, and the glycogen reprecipitated; finally, the hydrolysis of the glycogen is carried out by the method of Sjögren *et al.*[6]

The method of Folin and Wu[7] was used for the determination of glucose in the hydrolysate, and the amount of glycogen was calculated by means of the so-called Pflüger factor (0.927).[8] The difference in readings between the aliquots did not exceed 4% of their mean. The analytical results are recorded in Column 7 of Table I.

For the cytochemical preparations, the fixed blocks were embedded in 56-58°C paraffin in routine fashion; sections were carefully cut on a sliding microtome at 5 μ and stained for glycogen by the Bauer-Feulgen reagent following hydrolysis for one hour in 4% chromic acid.[9] After rinsing they were lightly counterstained with Harris' hematoxylin.

Estimations of the relative amounts of glycogen in the stained sections were initially tried by simple histological inspection of the intensity of staining. However, we have found a more satisfactory method for placing the preparations in order of glycogen content in the direct comparison of pairs of slides viewed simultaneously through a comparison ocular.

In order to obtain quantitative estimates of the glycogen content of the sections, 2 methods were employed. In the first the overall color of the section was compared with a standard solution of recolorized fuchsin by utilizing a comparison ocular attached to the eyepieces of the microscope and a Duboscq colorimeter.[10] This method proved only partly satisfactory since some difficulty was experienced in arriving at a reliable end-point.

The second quantitative method, and the one by which the data reported in the paper were obtained, proved more reliable. The amount of light transmitted through the section was measured by means of an electronic light-meter (Photovolt photometer, model 512). The image of the section was projected at a magnification of 100× into the search unit of the photometer. A green filter (Wratten X) was employed to accentuate absorption by the fuchsin stain. The lighting was so arranged that the photometer scale recorded 100 when the light was passing through a clear slide and coverslip. The stained section was then interposed and the degree of light absorption recorded. Four readings, from different parts of the section, were taken for each preparation and averaged; from this figure was subtracted the average light-absorption by those sections exhibiting no glycogen detectable by the eye. The aver-

---

[4] Rossman, I., *Am. J. Anat.*, 1940, **66**, 277.

[5] Good, C. A., Kramer, H., and Somogyi, M., *J. Biol. Chem.*, 1933, **100**, 485.

[6] Sjögren, B., Nordenskjöld, T., Holmgren, H., and Möllerström, J., *Arch. ges. Physiol.*, 1938, **240**, 427.

[7] Folin, O., and Wu, H., *J. Biol. Chem.*, 1920, **41**, 367.

[8] Nerking, J., *Arch. ges. Physiol.*, 1901, **85**, 320.

[9] Bensley, C. M., *Stain Tech.*, 1939, **14**, 47.

[10] Dempsey, E. W., and Singer, M., *Endocrinology*, 1946, **38**, 270.

TABLE I.
Photometric and Chemical Determinations of and Histological Distribution of Glycogen in Livers of Rats Killed After a Known Feeding Time.

1	2	3	4	5	6	7	8	9
		Optical density of sections			G glycogen/100 g tissue			
Hr since fed	Rat No	l.l.	r.l.	avg	calculated	found	Diff.	Distribution in lobule
6	a	29.5	28.5	29	4.8	4.0	+0.8	even or p.c.
	b	30	37	33.5	5.5	5.1	+0.4	,, ,, ,,
	c	25.5	29	27.3	4.5	4.9	—0.4	p.c.
	d	27	25	26	4.3	4.5	—0.2	,,
					—	—		
					4.8	4.6		
12	a	31	28	29.5	4.8	5.0	—0.2	even or p.c.
	b	24	22.5	23.3	3.8	5.3	—1.5	,, ,, ,,
	c	35	18	26.5	4.3	5.0	—0.7	,, ,, ,,
	d	31	29	30	4.9	4.7	+0.2	p.c.
					—	—		
					4.5	5.0		
18	a	18	15	16.5	2.7	2.2	+0.5	c.c.
	b	17.5	16.5	17	2.8	3.1	—0.3	p.c.
	c	22	22	22	3.6	3.0	+0.6	,,
	d	23	20.5	21.8	3.6	2.8	+0.8	,,
					—	—		
					3.2	2.8		
24	a	0	3	1.5	0.2	0.5	—0.3	c.c.
	b	11	11	11	1.8	1.7	+0.1	,,
	c	7	3	5	0.8	1.0	—0.2	,,
	d	6	4.5	5.3	0.9	0.9	0.0	,,
					—	—		
					0.9	1.0		
48	a	1	2	1.5	0.2	0.03	+0.2	invisible
	b	3	0	1.5	0.2	0.02	+0.2	,,
	c	1	0	0.5	0.1	0.06	0.0	,,
	d	0	0	0	0.0	0.08	—0.1	few cells scattered in c. zone
				—	—	——	—	
				$s_1 = 328.7$	0.1	0.05		
						$s_2 = 53.9$	s.d.±0.52	
			$s_2/s_1 = 0.164$					

l.l. = left lobe.
r.l. = right ,,
p.c. = peripheral concentration.
c.c. = central ,,

s_1 = total avg opt. densities.
s_2 = total found glycogen.

age differences among the 4 readings were ±2. The maximum variation between readings was 12 for liver No. 12d l.l., in which the extreme readings were 25 and 37. The corrected average readings of optical density are listed as Columns 3 and 4 of Table I. It will be noted that in the livers of rats 48 a, b and c, corrected photometer readings of 0.1-0.2 were made, even though there was no glycogen visible on microscopic examination. Such readings are, however, without significance and represent the limit of error of the method.

Results. The livers appeared to be uniformly and completely fixed. Unlike the usual histological preparations for glycogen[1] the sections possessed no rim of cells containing more glycogen than those in the center of the block. Moreover, as in frozen-dried preparations, glycogen was scattered in small particles throughout the cytoplasm rather than being concentrated against one membrane (Maximow and Bloom[11]). The quality of fixation is illustrated in the photomicrographs (Fig. 1-4).

Like the mouse,[12] the rat exhibits a zonation in the distribution of glycogen in the liver lobule, and Column 9 of Table I shows that the zonation undergoes a similar sequence of changes during starvation. Glycogen is great in amount and either evenly dis-

---

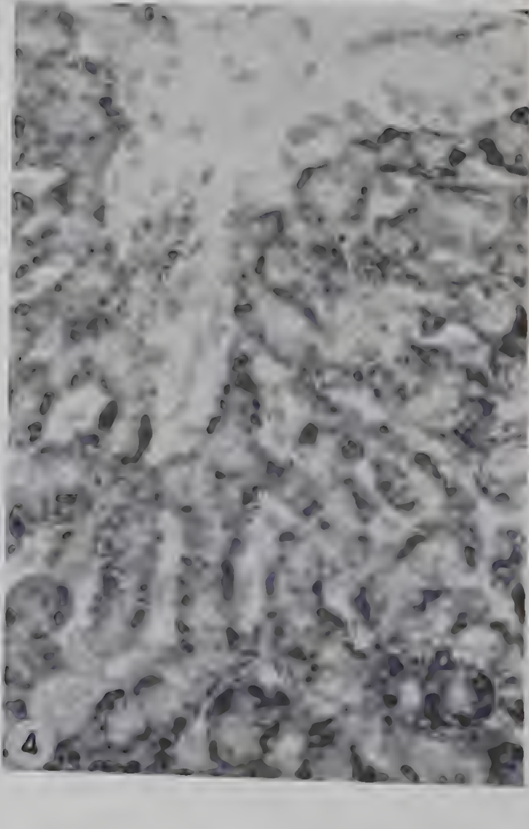
[11] Maximow, A. A., and Bloom, W., *A Textbook of Histology*, 1943, p. 15.

[12] Deane, H. W., *Anat. Rec.*, 1944, **88**, 39.

1

2

3


4

Photomicrographs of 5 μ sections of rat liver, fixed in ice-cold picro-alcohol-formalin and stained by the Bauer-Feulgen method for glycogen and lightly counterstained with Harris' hematoxylin. The pictures were taken with green and yellow filters combined (Wratten B and G) to accentuate the fuchsin color. They have all been printed on Kodabromide F1 paper to produce a comparable intensity of color.

FIG. 1. Rat 12d, left lobe of liver (glycogen content 4.7%, optical density 31). Marked concentration of glycogen, particularly in the peripheral zone of the lobule (above). The glycogen has not been "swept" across the cells by the method of fixation adopted here as is usual after fixation at room temperature. In the cells in the outer part of the lobule the greatest amount of glycogen lies adjacent to the sinusoids (See Fig. 4). ×120.

FIG. 2. Rat 18d, right lobe of liver (glycogen content 2.8%, optical density 23). Moderate amount of glycogen, about evenly distributed in the lobule but slightly more concentrated in the peripheral zone (above). The carbohydrate is evenly distributed in each cell although unevenly concentrated in different cells. ×120.

FIG. 3. Rat 24d, left lobe of liver (glycogen content 0.9%, optical density 6). Only slight amount of glycogen, occurring solely in the central part of the lobule (below). ×120.

FIG. 4. Rat 6a, right lobe of liver (glycogen content 4%, optical density 28.5). High power view of glycogen in cells near portal canal. It does not appear to have been displaced during fixation but to have been precipitated *in situ* (*cf.* Fig. 13 in Maximow and Bloom[11]). In peripheral cells glycogen is concentrated in the cytoplasm near the sinusoids. ×600.

tributed or peripherally concentrated in the lobule 6 and 12 hours after feeding (Fig. 1). It is reduced in amount and generally peripherally concentrated at 18 hours (Fig. 2), very low and centrally concentrated at 24 hours (Fig. 3), and virtually absent at 48 hours. Inspection of Table I shows that the shift from peripheral to central concentration occurs when the glycogen level is approximately 2%, and furthermore that the lowest amount of glycogen which is microscopically visible is 0.08%. This small amount of glycogen occurs in scattered cells only and is not detected by the photometric

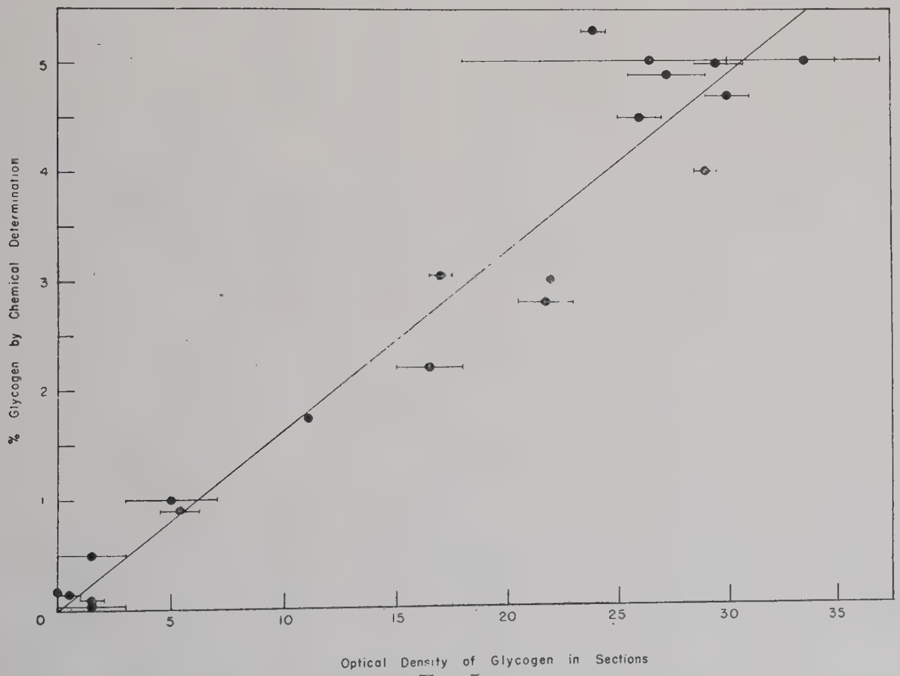


FIG. 5.

The black dots denote the chemically determined glycogen in a liver plotted against the optical density of the glycogen in the sections. The horizontal lines through the dots show the differences between the optical densities of the right and left lobes.

The diagonal line is satisfied by the equation: % Chemically Determined Glycogen = 0.164 × Optical Density.

method. Interestingly enough, when there is a large amount of glycogen in the cells near the periphery of the liver lobule, it appears to be most concentrated in the cytoplasm adjacent to the sinusoids (Fig. 1 and 4).

The appraisal of the amount of glycogen in histological preparations is hampered by the unequal zonation of glycogen in the liver lobule. It was also found that lobules of the same liver lobe may possess different quantities of glycogen. This is true even when the sections are entirely uniform in thickness. Moreover, the 2 lobes occasionally present significantly different levels of glycogen (*e.g.*, No. 6b, 12c, and 24c). The possibility of such differences in distribution needs to be taken into consideration by both histologists and biochemists in drawing conclusions from small samples of liver.

The analytical data (Column 7) and the average corrected photometer readings (Column 5) of the livers of the individual rats are recorded in Table I by groups, according to the lapse of time after the last feeding. The average ratio of glycogen found/photometer reading = 0.164. Multiplying the individual photometer readings by this factor gives a calculated value of the glycogen in the histological sections of each rat liver (Column 6). The standard deviation of the differences between the found and calculated glycogen values is ±0.52.

The data for chemically determined glycogen have also been plotted against the corrected photometer readings in Fig. 5. The black dots designate the average of the optical densities of the sections of a liver and the corresponding chemical glycogen determinations. The horizontal lines through the dots indicate the differences between photometer readings for the right and left lobes. It is seen that up to a glycogen concentration of about 5% a linear correlation exists between the 2 methods of determination.

It may be further pointed out that though no significant difference occurred in the liver glycogen between the 6- and 12-hour postfeeding periods, a marked progressive decrease occurred between the 12- to 18- and the 18- to 24-hour periods.

Summary. Glycogen of rat liver was well preserved throughout histological blocks and was not displaced in the cells when pieces of liver were fixed in ice-cold picro-alcohol-formalin. After fixation, liver sections were stained by the Bauer-Feulgen method and the optical density of each section was measured on a Photovolt electronic photometer. Chemical determinations were made of the glycogen contained in other pieces of the same livers. The livers of rats undergoing progressive starvation from 6 to 48 hours showed good correlation between the chemically and histologically determined glycogen concentrations.

15616 P

A Virus Causing Abnormal Milk in Cattle.

James A. Baker and Ralph B. Little. (Introduced by Carl TenBroeck.)

From the Department of Animal and Plant Pathology, The Rockefeller Institute for Medical Research, Princeton, N.J.

Dairy cows near Princeton, N. J., have shown an unusually high incidence of bloody thickened milk during the spring, summer and fall of 1946. The majority of cases occurred in the latter part of May and in June and animals in all stages of lactation were affected. Many specimens of abnormal milk contained flakes composed of polynuclear and mononuclear cells. Affected animals frequently showed temperatures exceeding 40°C but otherwise appeared normal and continued to eat as usual. In most cases all visible signs disappeared after 2 to 4 days. These facts suggested an infectious nature and investigations were made in an attempt to procure the etiological agent.

Guinea pigs weighing 200 to 300 g and kept under constant conditions of temperature (70°F) and humidity (60%) were inoculated intraperitoneally with either 1 or 5 cc of milk freshly drawn from naturally infected cows. From each of 3 milk samples furnished by different cows an agent was established in guinea pigs that caused temperature elevations exceeding 40°C beginning 4 to 7 days after inoculation and lasting for 3 days. Other than fever no definite signs of illness were noted, and all animals survived unless killed for further study. Animals killed during the febrile period showed hemorrhages in the lungs, focal necrosis of the liver and slightly enlarged mesenteric lymph nodes. These findings remained constant through 20 serial passages in guinea pigs maintained by blood transfers. Following recovery animals given a second inoculation with the same strain showed no response and in cross inoculations between the 3 strains it was found that each immunized against the others. Therefore presence of the agent was determined by a typical thermal response in guinea pigs, followed by subsequent immunity to test inoculation with a reference strain.

The agent was transferred from guinea pigs to rabbits by intravenous and to mice by intraperitoneal inoculation. Rabbits responded with a thermal reaction similar to that in guinea pigs. Mice showed no evidence of infection but tests showed that the agent was present after 3 serial passages. The agent was maintained for 1 but not 2 serial passages when inoculated into the yolk sac of 5-day embryonated hen's eggs. When inoculated on the chorioallantoic membrane of eggs that had been incubated for 10 days, tests showed that the agent was present after 10 serial transfers. No pathological alterations of the membrane were noted.

A lactating cow inoculated subcutaneously with 1 cc of blood from the first guinea pig transfer showed fever 7 days later, and a second lactating cow inoculated in the same way with blood from the 6th guinea pig passage showed fever on the 9th day. While both animals showed fever and alterations in the milk similar to those seen in natural cases, the milk did not contain red blood cells. The agent was demonstrated in the blood of both animals and in the milk of the first cow. Similar inoculations of the agent into 2 young calves produced a febrile reaction and tests showed that the blood contained the agent. Sera of animals recovered from either experimental or natural disease neutralized 100 to 1000 infectious doses for guinea pigs, whereas sera from normal animals and those in the acute phase of illness failed to neutralize 1 to 10 infectious doses.

Films from yolk sac membranes in the first passage and infected chorioallantoic membranes stained with methylene blue and by Macchiavello's method showed no organisms. Transfers of blood from infected guinea pigs and of infected chorioallantoic membranes showed no growth on blood agar slants and sealed cooked meat medium. This failure to demonstrate the agent microscopically or culturally and its specificity for the chorioallantoic membrane indicated that the agent was a virus.

From the work reported herein, it appears that a virus has been secured in guinea pigs from abnormal milk of diseased dairy cattle. When blood from infected guinea pigs was inoculated subcutaneously into lactating cows, a disease was produced resembling that seen in animals from which the virus came originally. Neutralizing antibodies developed in experimental cows and calves, and sera from cows that had recovered from the natural disease also neutralized the agent. Various workers have postulated that a virus may initiate some types of bacterial mastitis.[1] Further work must be done to determine whether this virus can act in such a manner.

---

[1] Little, R. B., and Plastridge, W. N., *Bovine Mastitis, A Symposium*, New York, McGraw-Hill Book Co., Inc., in press.

15617

Influence of Iodination on Proteolytic Activity and Blood Pressure Lowering Ability of Trypsin.*

DONALD E. BOWMAN.

From the Department of Biochemistry and Pharmacology, Indiana University School of Medicine, Indianapolis.

It has been pointed out by Silva[1] and by Dragstedt and his associates[2-5] that the intravenous administration of trypsin results in a decided fall in blood pressure, and attention has been called to the similarity between trypsin shock and anaphylactic shock.[6] During the course of observations dealing with the influence of intravenously-administered trypsin on the blood pressure of dogs, it was observed that the iodination of trypsin serves to decrease its influence on blood pressure with a smaller decrease in proteolytic activity.[7]

Methods. Crude trypsin was iodinated with 0.01 N or 0.1 N iodine in the presence of starch at 38°C with the solution buffered at pH 7 with phosphate buffer. The iodine was added in successive small quantities sufficient to maintain only slight to moderate starch-iodine color in the iodinating solution. The time required varied between 30 minutes and 4 hours, according to the degree of iodination. Following final decoloration the solutions were allowed to remain at 38°C for one additional hour and ascorbic acid was added as a precaution against the presence of free iodine. Control preparations were treated in exactly the same manner except for the addition of iodine.

The concentrations of starch, excess potassium iodide, and phosphate buffer, which are known to influence the rate of iodination, were maintained constant. Blood pressure recordings were made in the usual manner and complete recovery was allowed following each injection into the femoral vein.

Since the amount of substrate digested in a given period is not directly proportional to the concentration of the enzyme, the proteolytic activities of the various preparations were estimated by determining the relative amount of each preparation required to accomplish the same degree of casein digestion as a basic amount of the untreated enzyme under standardized conditions at pH 7 and 38°C. The undigested casein was precipitated with trichloracetic acid and was estimated by the Kjeldahl procedure.

TABLE I.
Influence of Partially Iodinated Trypsin on Blood Pressure.

Milliequivalents of iodine per mg of trypsin	Decrease in blood pressure upon injecting 1.5 mg of trypsin per kg, mm
0	62
0.2×10^{3}	55
0.4 ”	60
0.8 ”	39
1.2 ”	6
1.6 ”	7
2.0 ”	4
0	58

Results. The data of Table I indicate the blood pressure changes in a single dog following the injection of a series of trypsin preparations differing in degree of iodination. There is a decided decrease in the hypotensive effect with an increase in iodination. It was further observed that limited iodination which is insufficient to decrease the hypo-

---

* Read before the Federation of American Societies for Experimental Biology March 15, 1946.

[1] Rocha e Silva, M., *Arquivos d. Inst. Biol.*, 1939, **10**, 93.

[2] Ramirez de Arellano, M., Lawton, A. H., and Dragstedt, C. A., PROC. SOC. EXP. BIOL. AND MED., 1940, **43**, 360.

[3] Rocha e Silva, M., and Dragstedt, C. A., *J. Pharm. and Exp. Ther.*, 1941, **72**, 36.

[4] Dragstedt, C. A., and Rocha e Silva, M., PROC. SOC. EXP. BIOL. AND MED., 1941, **47**, 420.

[5] Rocha e Silva, M., and Dragstedt, C. A., PROC. SOC. EXP. BIOL. AND MED., 1941, **48**, 152.

[6] Dragstedt, C. A., and Wells, J. A., *Quart. Bull. Northwestern Univ. Med. School*, 1944, **18**, 104.

[7] Bowman, D. E., *Fed. Proc.*, 1946, **5**, 124.

TABLE II.
Influence of Large Doses of Iodinated and Non-iodinated Trypsin on Blood Pressure.

Dog No.	Milliequivalents of iodine per mg of trypsin	Amt of trypsin inj., mg/kg	Iodinated trypsin		Non-iodinated trypsin	
			Fall observed, mm	Duration of decrease, min	Fall observed, mm	Duration of decrease, min
10	1.6×10^{-3}	20	10	2	80	35
12	2.0 ”	20	32	2	80	fatal
14	2.0 ”	15	5	0.25	85	50
15	2.0 ”	20	10	1	70	40
16	2.0 ”	15	6	1	54	35
19	2.0 ”	15	11	1	62	25
17	2.0 ”	10	5	1	96	75

TABLE III.
Comparison of Proteolytic Activity and Hypotensive Effect.

Dog No.	Milliequivalents of iodine per mg of trypsin	Ratios of concentrations of iodinated to untreated trypsin required to obtain same proteolysis and hypotensive effect obtained with untreated trypsin	
		Proteolysis	Hypotensive effect
20	1.3×10^{-3}	2.5	30-35
21	1.3 ”	3.0	30-40
22	1.4 ”	2.0	30
23	1.5 ”	4.0	40
25	1.6 ”	3.5	45
26	1.7 ”	4.5	40-50
27	1.8 ”	4.5	40-60
28	1.9 ”	5.0	50-60
29	2.0 ”	6.0	60
30	2.0 ”	5.0	30

tensive effect of 1.5 mg of trypsin per kg of body weight does, however, serve to decrease the effect of smaller amounts of trypsin such as 0.5 mg per kg.

The data given in Table II show that the administration of relatively large doses of extensively iodinated trypsin does not cause the profound hypotensive changes noted with the same amount of untreated trypsin. In all cases, the iodinated enzyme was injected before the noniodinated and the blood pressure was allowed to return to the normal level before the second injection. The injections were intentionally made rather rapidly in 30 to 75 seconds according to the size of the dose and at about the same rate in each case.

The data presented in Table III refer to the results of the simultaneous comparison of proteolytic activity and hypotensive effect of each of 10 different preparations. The decrease in hypotensive activity of trypsin is accompanied by a smaller decrease in proteolytic activity. In each case the severity of shock resulting from 1 mg of untreated trypsin per kg was first determined by several injections, allowing for complete recovery after each injection. The iodinated preparations were then administered in increasing amounts until a hypotensive effect comparable, in degree of fall and duration, to that resulting from the untreated trypsin was obtained. Following this 1 mg per kg of the untreated enzyme was again administered and it was found that the final hypotensive effect was consistent with that produced originally by this amount. The untreated trypsin was then injected in amounts equal to the largest amount of the iodinated enzyme used and this invariably resulted in decided shock lasting for a period of 20 to 50 minutes, being fatal in some cases.

At lowered pH levels, such as pH 5, the iodination of trypsin is considerably retarded.

The amount of iodine taken up at this pH in a period of time sufficient to iodinate the enzyme extensively at pH 7 does not cause appreciable lowering of proteolytic activity or hypotensive effect. Likewise, the pH influences the rate of iodination of dilute solutions of histidine and tyrosine. Under conditions similar to those maintained for the iodination of trypsin, the time required for 1×10^{-4} millimoles of tyrosine to decolorize 2 equivalents of iodine per mole increases from 1 minute at pH 7 to 480 minutes at pH 5. The comparable values for histidine under the same conditions were found to be 17 minutes at pH 7 and more than 3000 minutes at pH 5. This suggests further consideration of these groups in the iodination of trypsin, with possible emphasis on tyrosine.

Iodination of a substrate rather than the enzyme, under similar conditions, decreases the proteolytic activity of untreated trypsin. Thus, the digestion of casein which has reacted with 2×10^{-3} milliequivalents of iodine per mg may be limited to about 45% of that of untreated casein. Furthermore, it has been observed that the iodination of an active protein substrate such as insulin serves to increase its resistance to trypsin. If the iodination is limited so that the iodinated hormone retains about one-half (52%) of its original activity it tends to resist digestion and further inactivation (to 44% of the original activity) by trypsin under conditions which markedly reduce the original activity of noniodinated insulin to 3%.

Summary. Iodination serves to decrease greatly the hypotensive effect of intravenously-administered trypsin. Proteolytic activity is decreased to a lesser extent. Iodination of a substrate under similar conditions decreases the ease with which it is digested by untreated trypsin.

15618

Production of Ketosis in Alloxan Diabetic Rats with Nicotinic Acid.

R. G. JANES AND L. MYERS.

From the Department of Anatomy, State University of Iowa, Iowa City, Iowa.

There is much evidence to support the belief that niacin is an important constituent of the enzyme systems of the body[1,2] although the reported effects of this vitamin on tissue metabolism and its co-activity with other vitamins lack clarity in certain instances.

Nicotinic acid appears to play a minor role in fat metabolism, resulting in an increase in body fat;[3] in a reduction of the lipotropic effect of choline;[4] and in an increased fatty infiltration of the liver in rats.[5]

The role of niacin in carbohydrate metabolism has been established largely by observations made on glycemic changes in the intact animal. Whether the administration of this vitamin produces hyperglycemia,[7-9]

---

[1] Warburg, O., and Christian, W., *Biochem.*, 1936, **287**, 291.

[2] Axelrod, A. E., and Elvehjem, C. A., *Nature*, 1939, **143**, 281.

[3] Gavin, G., and McHenry, E. W., *J. Biol. Chem.*, 1940, **132**, 41.

[4] Forbes, J. C., *J. Nutrition*, 1941, **22**, 359.

[5] Foà, P. P., Foà, N. L., and Field, H., Jr., *Arch. Biochem.*, 1945, **6**, 215.

[6] Scotti, G., *Riforma med.*, 1939, **55**, 1356.

[7] Morelli, A., and Greco, D., *Arch. sci. biol.*, 1939, **25**, 506.

[8] Morelli, A., and Olivia, F. P., *Arch. sci. biol.*, 1942, **28**, 279.

[9] Fichera, G., and Vasta, S., *Biochim. terap. sper.*, 1941, **28**, 100.

[10] Göbell, O., *Klin. Wchnschr.*, 1940, **19**, 710.

[11] Ledrut, J., *Bull. soc. chim. biol.*, 1940, **22**, 321.

[12] Cherkes, L. A., and Rozenfel'd, E. L., *Biokhimiya*, 1941, **6**, 58.

[13] Marche, J., and Delbarre, F., *C. R. soc. biol.*, 1943, **137**, 153.

[14] Boldyreff, E. B., *J. Am. Med. Assn.*, 1944, **124**, 257.

hypoglycemia[10–14] or is without effect on blood sugar levels,[6] are controversial points still under consideration.

In view of the evidence that niacin influences both fat and carbohydrate metabolism, alloxan diabetic rats were selected as subjects for this study since they are usually on the borderline of ketosis and respond more readily to any treatment that alters carbohydrate metabolism than do normal animals.

Experimental. Twenty adult rats of the Long-Evans strain, 2 females and 18 males, varying in weight from 235 to 400 g, were used in this study. These animals were severely diabetic following intraperitoneal injections of alloxan (125 mg/kg), although there was some variation in the degree of diabetes exhibited by individual rats. Three animals showed evidence of acidosis during the preliminary study period. The rats were checked to make certain the diabetes was stable 2 to 4 months after the onset of diabetes.

During the stabilization period the diabetic animals were fed a synthetic diet in which yeast (Pabst) was the source of the vitamin B complex. In the experimental period, a basic diet was fed in which crystalline B vitamins* were supplied as shown in the following formula:

BASIC DIET

Casein	180 g
Sucrose	300 ”
Starch	390 ”
Crisco	50 ”
Wesson Oil	50 ”
Haliver Oil	2 cc
Salt Mixture†	30 g
Choline	1 ”
Thiamin Hydrochloride	5 mg
Niacin	10 ”
Pyridoxine	5 ”
Calcium Pantothenate	15 ”
Riboflavin	5 ”

The experimental diet high in niacin was a modification of the basic diet containing 1 g niacin/kg instead of 10 mg niacin/kg. The length of time that the animals were allowed to remain on the basic or high-niacin diet was altered during the course of the investigation. The rats ate *ad libitum* and were kept in metabolism cages.

All determinations were made on nonfasting animals. Weights were taken every other day; food intake and urine volume were measured daily. Urine was collected under a few drops of toluol for acetone and urinary sugar studies. Blood sugar and urine sugar determinations were made at 5- to 6-day intervals by Somogyi's micro-method.[15] Because of the high levels of both blood and urine sugar, the blood filtrate was diluted 1:1 with distilled water, and only 1 cc of a 1:500 urine dilution was used in these determinations. No corrections were made for the nonfermentable substances in the urine

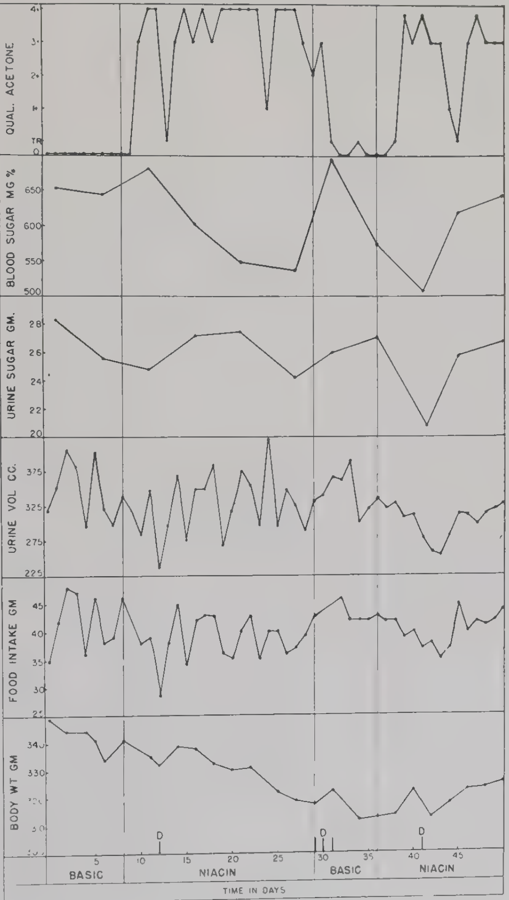


Fig. 1.
Data on rat with Type I ketonuric response. Tr. = trace. D = days when the rat had diarrhea.

---

* Crystalline vitamins supplied through the courtesy of Dr. J. R. Bright, Gelatine Products Corp.

† Hubbell, R. B., Mendel, L. B., and Wakeman, A. J., *J. Nutrition*, 1937, **14**, 273.

[15] Somogyi, M., *J. Biol. Chem.*, 1945, **160**, 69.

since preliminary tests proved them to be negligible. Acetone bodies were estimated daily by the sodium nitroprusside test. Quantitative total urinary acetone body determinations were made for several of the animals at 2- to 5-day intervals by the gravimetric method of Van Slyke.

Results. Eighty % of the diabetic animals fed on high-niacin diet exhibited ketosis of a pathological degree. Four rats showed ketosis during the first 25 hours after the high-niacin diet was fed; 3 during the 2nd day; 2 on the 3rd day; one on the 4th day; 4 on the 5th day; one on the 7th day; and one on the 20th day.

No uniform response pattern of ketosis was exhibited, although most of the rats developed ketonuria in 1-5 days after high-niacin diet was given. The severity and duration varied from animal to animal. The rats were grouped according to their particular ketonuric response to niacin, or their failure to show symptoms of ketosis:

(1) Eight rats which showed ketonuria only while they were on the high-niacin diet. Some of these rats responded rapidly (Fig. 1) while others were slow to reach their peak of ketosis (Fig. 2). Acidosis was decidedly positive as long as these animals were ingesting niacin, but as soon as the niacin was withdrawn it disappeared, becoming evident again when the high-niacin diet was reinstated.

(2) Five rats which developed ketosis on the high-niacin diet, all had a remission before niacin was withdrawn (Fig. 3). Of this group, 3 failed to exhibit ketonuria on the second introduction of high-niacin, made after a period on basic diet.

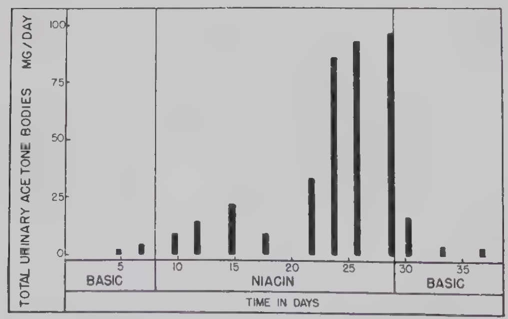


FIG. 2.
Urinary acetone bodies from rat that responded slowly to niacin. Type I response.

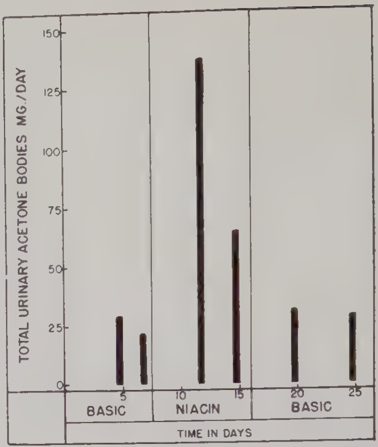


FIG. 3.
Type II response. Rat had mild ketosis before niacin was given; and showed a remission before niacin was withdrawn.

(3) Three rats showed ketonuria while they were on the high-niacin diet, became negative when placed on basic, then developed temporary ketonuria 5 to 7 days afterward, while still on the basic diet.

(4) Four animals were resistant to the formation of excess ketone bodies when placed on high-niacin diet. These rats were severely diabetic, as indicated by the nonfasting blood sugar levels which ranged from 500-650 mg %, and should have been just as vulnerable to acidosis as the other experimental animals.

Niacin intake per 100 g of body weight was calculated for all rats which showed ketonuric response and for those which did not. There seemed to be no relationship between the amount of niacin ingested and the degree of ketonuria exhibited.

The weight of the rats and their food intake were followed closely throughout the experimental period. The animals, particularly those which were most severely diabetic, lost weight during the course of the experiment. This was not unusual since the animals were not treated with insulin. The food intake did not change materially (Fig. 1), either on basic or high-niacin diet, although there was considerable variation from day to day.

The urine volume fluctuated in proportion to the food intake and varied in different groups of animals from an average minimum of 90 cc to an average maximum of 360 cc daily.

Although there was some fluctuation throughout the experiment in levels of blood sugar and in quantities of urinary glucose excreted (Fig. 1) by a single animal, these were directly correlated, in most cases, with the food intake. That is, when the food intake increased, there was a corresponding increase in blood sugar and in urinary glucose excretion. A factor of importance in considering blood sugar fluctuations is that determinations were made on nonfasting animals, in which the time between last ingestion of food and withdrawal of blood for glucose determinations was not controlled.

Discussion. Normal rats are relatively resistant to ketosis. Before urinary acetone bodies appear in appreciable quantities, rats must either undergo long periods of fasting,[16] or high-fat diets must have been previously ingested if shorter periods are to be effective.[17,18] Rats which have been diabetic with optimum amounts of alloxan are generally free from excessive ketone bodies after they have recovered from the immediate effect of this drug. However, these rats are always on the borderline of ketosis when they are severely diabetic.

In the present experiment, ketosis has been produced in 16 of 20 diabetic rats by means of a diet high in niacin. Although the animals showed a somewhat variable pattern, the degree of urinary ketone excretion was great enough to indicate that niacin ingested in large quantities produces some alteration in fat metabolism.

It has been shown that the introduction of relatively large amounts of niacin or niacinamide will cause a depletion of the labile methyl supply in the body,[19,20] as these groups are necessary to the formation of trigonelline (the methyl betaine of nicotinic acid) which is excreted in the urine of the rat after niacin has been fed. In correlating the observed increase in urinary acetone bodies with the intake of large amounts of niacin, it seems possible that the synthesis of choline, requiring methyl groups, is blocked or retarded by the reduction of free methyl groups coincident with high niacin intake. A choline deficiency would, in such a case, become the limiting factor in normal fat metabolism which would then result in an increase of total liver fat deposition and in the production of excessive ketone bodies.

To test this hypothesis, choline was given in large amounts (3 g/kg in certain diets) without altering the ketonuric response. Therefore, it has been concluded that the ketosis observed in this experiment is not caused by the lack of dietary choline.

When total blood fat was determined in some instances, there was no apparent difference between ketonuric and nonketonuric rats. Furthermore, a few observations indicated that the livers of animals which exhibited ketonuria contained no more total fat than those which were free from ketonuria. Lipoid distribution, however, as evidenced in osmic acid preparations, was clumped and sparse in livers of nonketonuric rats but was diffuse and rather fine in livers of ketonuric animals (unpublished data).

Certainly the increased ketogenesis which has been noted in the present experiment was not the result of a decreased food intake. The rats were allowed to feed *ad libitum* and the food intake remained fairly constant whether they were feeding on basic or high-niacin diet. There was no evidence of an alteration in carbohydrate metabolism consequent to the high niacin intake that could be detected from urinary and blood sugar studies.

Summary. A series of 20 rats of the Long-Evans strain which had severe alloxan dia-

---

[16] Deuel, H. J., Jr., and Gulick, M., *J. Biol. Chem.*, 1932, **96**, 25.

[17] Deuel, H. J., Jr., and Hallman, L. F., *J. Biol. Chem.*, 1941, **140**, 545.

[18] Roberts, S., and Samuels, L. T., *J. Biol. Chem.*, 1943, **151**, 267.

[19] Handler, P., and Dann, W. J., *J. Biol. Chem.*, 1942, **146**, 357.

[20] Handler, P., *J. Biol. Chem.*, 1944, **154**, 203.

betes for a preliminary period of 2 to 4 months, was given a diet with excess nicotinic acid. The diet was adequate but contained 1 g niacin/kg. The most outstanding effect of this diet was the production of ketonuria in 16 of the 20 animals, although the degree of ketonuria and the response pattern was variable. Supplementary choline did not inhibit ketosis. The action of nicotinic acid in altering fat metabolism is discussed.

15619

Inadequacy of Certain Natural Diets for Guinea Pigs.*

MOTT D. CANNON, G. J. MANNERING, C. A. ELVEHJEM, AND E. B. HART.

From the Department of Biochemistry, College of Agriculture, University of Wisconsin, Madison.

Ross, Phillips and Bohstedt[1] found that a ration composed of corn, soybean oil meal and alfalfa, which is similar to rations widely used for growing hogs, is inadequate for reproduction and lactation in swine and rats. It was, therefore, of interest to try this ration with guinea pigs in order to determine if the deficiencies in these animals were related to the postulated factors required specifically by guinea pigs.

Experimental and Results. The methods of handling the animals and securing the blood samples have been described previously.[2] Young guinea pigs initially weighing 150 to 200 g were housed in individual screen-bottomed cages, and kept on experiment for 6 weeks.

The basal ration (R-1) consisted of ground yellow corn 75 g, soybean oil meal 17.5 g, ground alfalfa 5 g, and salts 2.5 g. ($CaHPO_4$ 1.18 g, $CaCO_3$ 0.70 g, NaCl (iodized) 0.60 g, $MnSO_4$ 0.03). When supplements were incorporated into the ration, the amount of corn was correspondingly decreased. The letter *c* following the ration number indicates that the ration contained crystalline vitamins in the amounts used in experiments with purified diets:[2] thiamine 0.3 mg, riboflavin 0.5 mg, pyridoxine 0.4 mg, Ca *d*-pantothenate 1.5 mg, niacin 5 mg, choline chloride 100 mg, biotin 0.02 mg, *i*-inositol 50 mg, and 2-methyl-1,4-naphthoquinone 0.2 mg. All animals were given 20 mg of ascorbic acid orally every other day and 1 mg of α-tocopherol and 0.05 cc of haliver oil orally twice a week. The solution of α-tocopherol in corn oil was mixed with the haliver oil just prior to feeding.

The results obtained with Ration R-1 with and without various supplements are summarized in Table I. The results for the group receiving stock ration, a commercial-pelleted guinea pig feed, were described in a preceding publication[2] and are included for comparison. The animals that received Ration R-1 grew very poorly and less than half of them survived the 6-week experimental period. These animals showed a moderate anemia and leucopenia.

The addition of the crystalline vitamins (R-1c) enabled all animals to survive the experimental period and to maintain essentially normal levels of blood elements with a rate of growth ⅓ to ½ that of animals receiving the stock ration. With the work of Krehl, Teply, Sarma and Elvehjem[3,4] in mind, the vitamin mixture was replaced by

---

* Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. This work was supported in part by grants from the Cerophyl Laboratories, Inc., Kansas City, Mo., and the Research Funds of the University.

[1] Ross, O. B., Phillips, P. H., and Bohstedt, G., *J. Animal Sci.*, 1942, **1**, 86; 1942, **1**, 353.

[2] Cannon, M. D., Mannering, G. J., Zepplin, M., Elvehjem, C. A., and Hart, E. B., *Arch. Biochem.*, 1945, **7**, 55.

[3] Krehl, W. A., Teply, L. J., and Elvehjem, C. A., *Science*, 1945, **101**, 283.

[4] Krehl, W. A., Teply, L. J., Sarma, P. S., and Elvehjem, C. A., *Science*, 1945, **101**, 489.

TABLE I.
Growth and Blood Status of Guinea Pigs Fed a Natural Ration, With and Without Various Supplements.

Ration	No. of animals	Growth		Blood*	
		Avg, g per day for 6 wks	Range, g per day for 6 wks	Leucocytes, per mm³	Hemoglobin, g per 100 cc
Stock ration	15	7.5	5.0-10.2	5300	14.2
R-1	7	1.3	—1.1- 3.3	3900	11.8
R-1 + 0.01% niacin	4	2.7	1.8- 3.8	5400	12.1
R-1 + vitamin mixture (R-1c)	12	3.0	1.8- 5.1	6200	13.0
R-1c + 4% liver 1:20 powder	6	3.8	2.5- 6.6	7400	13.8
R-1c + 4% grass juice powder	3	3.9	3.1- 5.0	5700	13.1
R-1c + 15% extract wheat germ	3	4.2	4.1- 4.3	4700	12.5
R-1c + 10% linseed oil meal	3	4.8	4.1- 5.6	7100	11.2
R-1c + 25% ” ” ”	6	6.5	5.0- 7.5	7500	14.1
R-1c + ethanol extract l.o.m. ≅ 25%	3	3.5	2.7- 4.2	2900	12.5
R-1c + 25% l.o.m. residue	3	4.5	3.4- 5.4	8600	13.5
R-1c + l.o.m. extr. + residue ≅ 25%	3	6.7	5.4- 7.3	4700	13.5
R-1c + 25% soybean oil meal	3	5.6	5.0- 6.1	5300	12.8
R-1c + 8% Labco casein	3	4.9	3.6- 6.8	4700	14.3

* After 6 weeks on experiment.

a high level of niacin. Although growth and survival were better than on Ration R-1 alone, neither the growth nor the blood status was equal to that of animals provided with much smaller amounts of niacin, together with other vitamins.

The inclusion of 4% of 1:20 liver powder† or 4% of grass juice powder‡ in Ration R-1c gave little additional improvement in growth or blood formation. Extracted wheat germ (VioBin) at a level of 15% gave a small but definite improvement in growth, as did linseed oil meal at a 10% level. When 25% of linseed oil meal was added, blood formation was excellent and growth approached that of the animals fed the stock ration. Extraction of linseed oil meal with cold 50% ethanol (extract contained 3% of original l.o.m. solids) appears to remove some active substance, since growth with the rations containing the residue alone was inferior to that with whole linseed oil meal. The extract appeared ineffective by itself but when it was recombined with the residue growth as good as that with whole linseed oil meal was obtained. The addition of 25% of soybean oil meal (a total of 42% in the ration) supported good growth, although the average growth and hemoglobin values were lower than with linseed oil meal at 25%. The inclusion of 8% of Labco casein in Ration R-1c gave a definite average growth increase, though there was individual variation from 3.6 to 6.8 g per day. This average increase is probably significant since only 3 out of 12 animals on Ration R-1c alone showed gains in excess of 3.4 g per day. The blood picture was uniform and essentially normal.

The foregoing data indicate that Ration R-1 supplemented with vitamins A, D, E, and C is inadequate for growth and blood formation in guinea pigs. The deficiency may be made good in part by a mixture of the crystalline water-soluble vitamins of which niacin appears to be one, but not the only, active component. The blood status of animals receiving the vitamin supplement approaches that of animals fed the stock ration, although growth is still poor. Cunha *et al.*[5] have shown that added inositol is required with this ration to prevent alopecia in the rat and that inositol together with pyridoxine and a folic acid concentrate in-

---

† Dried crude water extract of liver.

‡ Grass juice powder is the spray-dried pressed juice of cereal grasses. One g of grass juice powder is equivalent to about 4 g of dried grass (Cerophyl).

[5] Cunha, T. J., Kirkwood, S., Phillips, P. H., and Bohstedt, G., PROC. SOC. EXP. BIOL. AND MED., 1943, **54**, 236.

TABLE II.
Effect of Tryptophane as Compared with Other Supplements on Growth and Blood Status of Guinea Pigs.

Ration	No. of animals	Growth: Avg, g per day for 6 wks	Growth: Range, g per day for 6 wks	Blood*: Leucocytes, per mm³	Blood*: Hemoglobin, g per 100 cc
Stock ration	13	5.2	3.0-6.7	6900	14.1
R-1c	8	1.9	0.5-2.8	5700	13.4
R-1c + 0.3% *dl*-tryptophane	4	3.4	3.0-3.8	7300	14.4
R-1c + ethanol extract l.o.m. ≅ 25%	4	2.0	0 -3.5	6000	13.2
R-1c + ethanol extract l.o.m. ≅ 25% + 0.3% *dl*-tryptophane	4	3.0	1.6-4.8	6600	14.2
R-1c + 25% l.o.m. residue	4	4.1	2.6-6.1	5400	13.9
R-1c + l.o.m. residue + extr. ≅ 25%	4	4.1	3.1-5.0	5100	14.5

* After 6 weeks on experiment.

creases the growth rate of the rat. Folic acid does not appear to be concerned here since 1:20 liver powder and grass juice powder, both rich in this factor, give only slight gains. The specific guinea pig factors postulated by ourselves and others[2,6,7] to be present in these materials are ruled out by this same evidence.

The substances which produced the greatest growth stimulation, linseed oil meal, soybean oil meal, and casein are protein-rich materials. Wheat germ which gave a slight response is likewise a good source of protein; 15% supplying 6% additional protein. The action of these substances does not appear to be due to the increase in the quantity of protein, since this is presumably adequate in the basal (Ration R-1, 15%, stock ration 17%), but to the addition of amino acids or other essential factors present only in limited quantity in Ration R-1. The studies of Krehl *et al.*[3,4] suggest that tryptophane may be a limiting factor with diets high in corn.

Assays employing *Lactobacillus arabinosus*[§] indicate that 100 g of Ration R-1 contains 0.20 g of tryptophane. Similar determinations show that the inclusion of 8% of casein supplies 0.10 g, 25% of soybean oil meal, 0.17 g, and 25% of linseed oil meal, 0.24 g of additional tryptophane per 100 g of ration. Calculations from the tables of Block and Bolling[8] indicate that 15% of wheat germ, which gave relatively little growth stimulation, adds only 0.06 g of tryptophane. Thus the growth stimulating potency of these 4 supplements appears to be directly proportional to their tryptophane content. The most effective of these, linseed oil meal, essentially doubles the amount of tryptophane in the ration. Apparently little or no tryptophane is destroyed or extracted from linseed oil meal by cold 50% ethanol since the residue contains 10.0 mg per g and the extract 2 mg per g (original linseed oil meal 9.5 mg per g).

In view of the above results we decided to test the effect of adding pure tryptophane. Unfortunately, no animals from our stock colony were available so it was necessary to use guinea pigs purchased from a commercial breeder. The animals as received from the breeder were given stock ration for a few days, then distributed at random among the groups receiving the R-rations listed in Table II. A group of animals from this same breeder received stock ration and the results are also included for comparison. All animals showed a slower rate of growth, regardless of the ration fed. This is made clear by comparing the growth of the groups fed stock ration in Tables I and II.

---

[6] Woolley, D. W., *J. Biol. Chem.*, 1942, **143**, 679.

[7] Woolley, D. W., and Sprince, H., *J. Biol. Chem.*, 1944, **153**, 687.

[§] The authors are indebted to Irene Greenhut, through whose cooperation these determinations were made.

[8] Block, R. J., and Bolling, D., *The Amino Acid Composition of Proteins and Foods.*

The addition of 0.3% of *dl*-tryptophane to Rations R-1c and R-1c + linseed oil meal extract stimulates growth and possibly increases the hemoglobin level (Table II) although it does not seem to duplicate completely the effect of 25% of linseed oil meal residue plus extract. In this connection it is interesting to point out that Woolley and Sprince[7] have shown that the dietary essential for guinea pigs designated at GPF 2 and found in linseed oil meal residue can be replaced by a mixture of cellulose and casein or cellulose, arginine, cystine and glycine. Apparently the guinea pig is susceptible to certain amino acid deficiencies and the limiting amino acid is dependent upon the kind of ration used. Since we have obtained excellent growth and development on a stock ration containing only 17% total protein it is evident that the amino acid requirement of guinea pigs is easily influenced by the remaining components of the diet.

Linseed oil meal extract does not seem to potentiate the action of tryptophane, nor in this series did it increase the growth stimulus supplied by 25% of linseed oil meal residue. Although growth on the linseed oil meal supplemented rations was much poorer than in the series reported in Table I, it should be noted that the addition of linseed oil meal does allow growth almost equal to that of animals from the same source receiving stock ration.

Summary. Guinea pigs fail to grow on a corn-soybean oil meal-alfalfa ration even when adequate amounts of vitamins A, D, E, and C are supplied. The survival and blood picture of the animals are improved by the addition of a mixture of crystalline water soluble vitamins of which niacin appears to be an important, but not the only, active component. Linseed oil meal, soybean oil meal, and casein supplements promote growth and blood formation approaching that of animals on stock ration. The potency of these substances is proportional to their tryptophane content and may be replaced in part by pure tryptophane.

We are indebted to Merck and Company, Inc., Rahway, N.J., for the synthetic vitamins; to the Abbott Laboratories, North Chicago, Ill., for haliver oil; and to the Wilson Laboratories, Chicago, Ill., for the various liver products.

15620 P

Effects of Androgen upon Reproductive Organs of Normal and Castrated Fetuses with Note on Adrenalectomy.*

L. J. Wells.†

From the Department of Anatomy, University of Minnesota, Minneapolis.

This report deals with progress of studies designed to determine whether in placental mammals the normal, prenatal development of the reproductive organs is influenced by hormones. Recently it has been found that in unborn rats the number and size of the interstitial cells of Leydig may be increased experimentally by injecting gonadotrophin under the skin of the unborn.[1]

In the present experiments the animals used were rats in which pregnancy was dated from the time of observation of copulation. Fetuses were killed at about one hour preceding the estimated time of normal parturition (21 days and 16 hours after copulation). After weighing a fetus, its pelvic region was fixed in Bouin's solution and sectioned serially at 10 μ. The sections were stained with hematoxylin and eosin or with

---

* Aided by grants from Ciba Pharmaceutical Products, Inc., and from the medical research funds of the Graduate School.

† With the technical assistance of Mrs. Dorothy N. Highby.

[1] Wells, L. J., Proc. Soc. Exp. Biol. and Med., 1946, **62**, 250.

TABLE I.
Volume of Reproductive Glands per Unit of Body Weight.*

Fetuses			Glands	
Groups	Treatment	Autopsy, hr after operation	Seminal vesicle, $(mm^3/g) \times 100$	Bulbo-urethral, $(mm^3/g) \times 1000$
A	None (controls)		.18 (18)	.27 (20)
B	Androgen (given to mothers)		.24 (11)	.45 (11)
C	Castration (Method 1)	20-61	.10 (6)	.17 (5)
D	Castr. (Method 1) and adrenalectomy	36-51	.09 (5)	.14 (5)
E	Adrenalectomy	51-61	.19 (3)	.28 (3)
	Fetuses of 6 litters.			
F	None (controls)		.17 (5)	.26 (5)
G	Castration (Method 2)	51-52	.11 (6)	.18 (6)
H	Castr. (Method 2) and androgen (pellet)	51-52	.21 (5)	.29 (5)
I	"Castration" (Method 3)	51	.12 (1)	.18 (1)

* Vertical columns in parentheses show number of fetuses from which the data were obtained.

alum cochineal and orange G. Volumetric determinations were made by paper-weight method of Hammar,[2] using the formula of Boyden.[3] All sections of a gland were projected at a magnification of 200 diameters. The epithelium of each section was drawn by outlining the basement membrane. In Groups A to E (Table I) the "volume of the seminal vesicle" is actually that of the gland, of the ejaculatory duct and of the proximal portion of the ductus deferens (portion to point where right and left tubes begin to diverge). In Groups F to I it is the volume of the gland and of the ejaculatory duct.

To investigate the effects of androgen upon the reproductive organs of normal male fetuses, 13 mothers were given subcutaneous injections of testosterone propionate.‡ Injections were made daily on days 13 to 22 of pregnancy, the daily dose being 15, 10 or 5 mg (Group B). It was found that at autopsy the fetuses of injected mothers were smaller than those of controls. The data suggest that the androgen had stimulated growth of the seminal vesicles and the bulbo-urethral glands. Although Greene, Burrill and Ivy failed to note any effects of androgen upon the reproductive organs of male fetuses, their studies did not include volumetric determinations.[4]

In order to determine whether the accessory reproductive organs are influenced by hormones produced by certain fetal glands, fetuses were subjected to laparotomy[5] and deprived of their testes by severing the *gubernaculum testis* and the *ductus deferens* (inguinal incisions, Groups C and D). Adrenals were removed through oblique lumbar incisions (Groups D and E). Since the volume of the test organs of Groups C and D was much smaller than that of controls, the question arises as to whether this was due to surgical alteration of the blood supply of these organs incidental to castration.

That this was not the case is suggested by the data on test organs of fetuses castrated by carefully shelling the testes out of the epididymides (Method 2, Group G). Similarly, it is suggested by the data obtained from a fetus "castrated" by shelling out the contents of the tunica albuginea (Method 3, Group I).

That the accessory reproductive organs of castrated fetuses were small because they had been deprived of a hormone produced by the testes (androgen) is suggested by the data on castrated fetuses in which, at the time of castration, a pellet of testosterone propionate was implanted under the skin of the back (about 1 mg). Hormone from the pellet could reach the test organs only provided they still had an adequate blood supply.

---

[2] Hammar, J. A., *Z. f. angew. Anat. u. Konstitutionslehre,* 1914, **1**, 312.

[3] Boyden, E. A., *Carnegie Inst. Wash., Contrib. to Embryol.,* 1940, **28**, 157.

‡ Perandren, supplied by Ciba Pharmaceutical Products, Inc., through the courtesy of Ernst Oppenheimer, M.D.

[4] Greene, R. R., Burrill, M. W., and Ivy, A. C., *J. Exp. Zool.,* 1941, **87**, 211.

[5] Wells, L. J., *Anat. Rec.,* 1946, **94**, 530.

Preliminary data on the number of mitoses in transverse sections of the seminal vesicle were obtained by counting the mitotic figures in the last 10 sections preceding junction of seminal vesicle and ductus deferens to produce ejaculatory duct. In Groups F to I the averages were, respectively, 20.5, 8.1, 17.0 and 12.0.

Pending additional data, it would seem that in this species the fetal testes produce a hormone which accelerates the growth of such organs as the seminal vesicles and the bulbo-urethral glands. If the adrenals of fetal males produce any such hormone, the quantity is not sufficient to prevent the effects of castration upon the accessory reproductive organs.

15621

Osmotic and Mechanical Fragilities of Erythrocytes of Monkeys Infected with *P. knowlesi* Malaria.*

Shu Chu Shen, Eleanor M. Fleming, and W. B. Castle.

From the Thorndike Memorial Laboratory, the Second and Fourth Medical Services (Harvard), Boston City Hospital, and the Department of Medicine, Harvard Medical School.

The objective of the experiments reported here was (a) to determine the osmotic and mechanical fragilities of the red blood cells of monkeys infected with malaria and (b) to compare the osmotic fragilities of the red blood cells before and after trauma.

Methods. Samples of the freshly drawn oxalated blood of 7 *Macaca mulatta* monkeys experimentally infected with *P. knowlesi* 5 to 9 days previously and of 6 uninfected control monkeys were studied.† For the determination of the osmotic fragility of the red blood cells, 0.1 cc of blood was introduced into 1 cc of a series of progressively hypotonic concentrations of NaCl and into 1 cc of distilled water. Approximately 10 minutes later these samples were centrifugalized at 1500 r.p.m. for 10 minutes. The percentage concentration of hemoglobin in each supernatant was then determined by appropriate use of the Evelyn photoelectric colorimeter, taking that in the tube containing distilled water as a concentration of 100%. A smooth curve was then drawn connecting the points representing the percentages of hemoglobin liberated at each tonicity. From this were read off the respective concentrations of NaCl required to liberate the characteristic percentages of hemoglobin shown in Table I.

The mechanical fragility of the red blood cells was determined according to a recently published method.[1] One-half cc samples of blood with hematocrits adjusted to about 40% were traumatized by glass beads rolling in the greatest internal circumference of 50 cc rubber-stoppered Erlenmeyer flasks, rotated about a horizontal axis perpendicular to their flat base. The percentages of hemoglobin liberated by 1½ hours of such trauma were considered to represent the mechanical fragility of the samples.

Usually an infected and a normal control sample were studied simultaneously. The initial osmotic and mechanical fragilities of a portion of each sample were determined. If there was sufficient blood, the osmotic

---

* This investigation was aided by a grant from the John and Mary R. Markle Foundation.

† The blood samples were obtained through the kindness of Dr. Quentin M. Geiman from animals being studied under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Harvard University.

[1] Shen, S. C., Castle, W. B., and Fleming, E. M., *Science*, 1944, **100**, 387.

TABLE I.

Characteristics of Blood of Infected Monkeys.

				Mechanical fragility		Intracellular parasites			% NaCl causing % hemolysis indicated							
Exp. No.	Time, hr	R.B.C., mils.	Hematocrit, %	Hematocrit (adjusted) %	Hemolysis %	Trophozoites %	Schizonts %	Total, %	1	2.5	5	10	50	75	95	
44-109	0	5.05	40.3	40.3	6.3	2.7	1.8	4.5	.52	.49	.47	.45	.39	.36	.32	
44-112	0	4.22	31.0	39.0	8.6	1.2	5.8	7.2	.68	.59	.48	.43	.38	.35	.28	
44-119	0	2.90	20.0	44.0	8.3	2.7	12.3	15.0	.72	.61	.55	.47	.37	.34	.30	
	9					1.0	4.0	6.2	.72	.66	.58	.45	.34	.31	.26	
	9					0.4	1.8	2.2	.54	.47	.42	.41	.36	.33	.29	Rotated
44-128	0	3.43	27.0	39.0	8.7	0.2	22.4	22.6	.62	.58	.53	.47	.35	.33	.26	
	9					1.0	25.3	26.3	.84	.81	.70	.58	.35	.34	.26	
	9					0.2	8.6	8.8	.78	.70	.60	.43	.34	.31	.25	,,
44-134	0	2.35	18.0	44.4	23.3	0.7	32.3	33.0	.74	.72	.68	.60	.39	.35	.28	
	9					0.2	28.8	29.0	.84	.82	.79	.70	.43	.36	.27	
	9					0.0	3.4	3.4	.59	.57	.54	.52	.42	—	—	,,
45-7	0	2.70	25.1	32.7	12.8	10.0	41.0	51.0	.81	.77	.74	.68	.48	.43	.37	
	9					2.4	15.6	18.0	.89	.88	.85	.80	.49	.44	.37	
	? < 9					9.0	14.2	23.2	.84	.82	.80	.70	.47	.41	.31	,,
45-57	0	2.08	16.0	40.2	15.0	22.7	24.7	47.4	.77	.73	.68	.60	.37	.33	.21	
	9					14.2	13.0	27.2	.87	.82	.73	.62	.37	.32	.21	
	9					14.7	2.8	17.5	.64	.59	.54	.49	.37	.33	.22	,,
Average Characteristics of Blood of Normal Monkeys.																
6 animals	0		43.5	43.5	3.8	0	0	0	.47	.44	.42	.41	.36	.34	.28	
3 animals	9								.49	.44	.41	.38	.34	.31	.24	,,

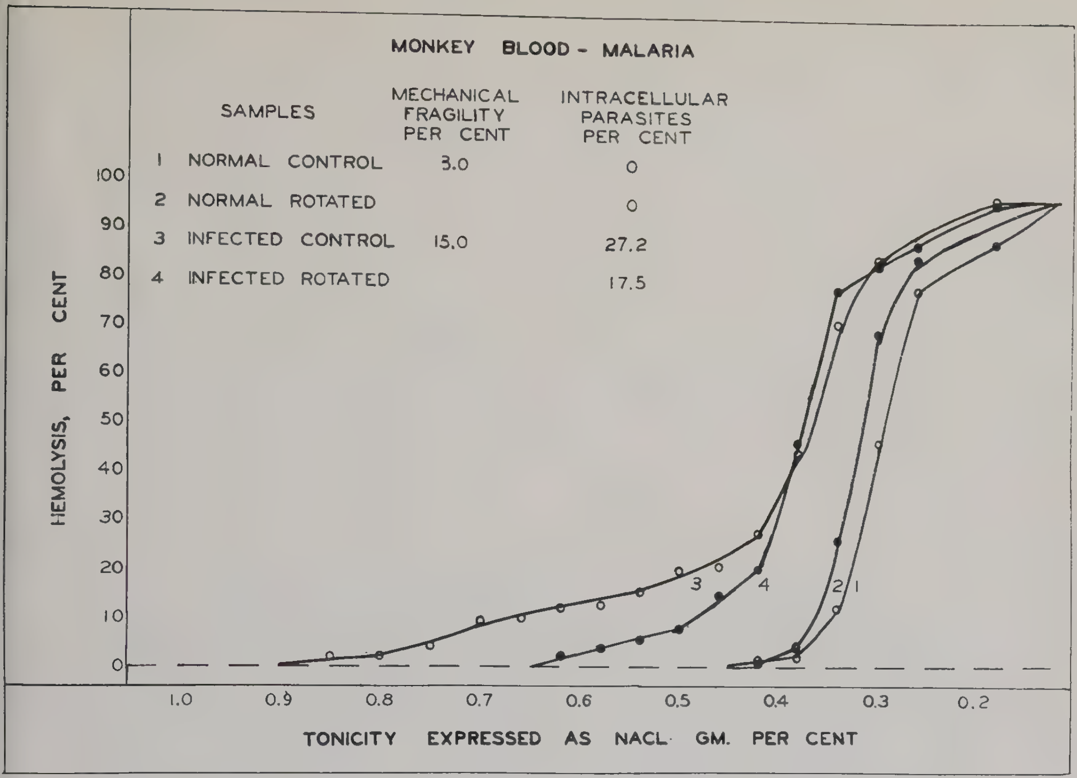


FIG. 1.

Effect of trauma (rotation with glass beads) on the osmotic fragility and percentage of parasitized red blood cells of a monkey with malaria (Exp. 45-57). The osmotic fragility of the red blood cells of this normal control monkey is less than that of the average normal monkey (Table I).

fragility of another portion of each sample was also determined after 9 hours of rotation with glass beads at room temperature. As controls, portions of each sample were also allowed to stand for 9 hours at room temperature and the osmotic fragility again ascertained. In addition, the percentages of red blood cells containing trophozoites and schizonts and their total were determined from blood films stained by Wright's method. Such parasite counts were made from the infected blood samples before and after the 9-hour period of mechanical trauma as well as from the portions of the samples which had stood for 9 hours at room temperature.

Results. Inspection of the data shown in Table I indicates that, as previously observed by Geiman and his associates,[2] the blood of all of the infected monkeys exhibited greater osmotic fragility of a portion of the red blood cells than did that of the uninfected controls. Fig. 1 (Exp. 45-57) exhibits in curve 1 the S-shaped form of the curve of osmotic fragility of the control normal monkey blood although it is to be noted that the red blood cells of this monkey were generally less fragile than those of the average normal monkey (Table I). In contrast to this symmetrical curve is the "trailing" form of the left end of curve 3 representing the osmotic fragility of the infected monkey blood. This asymmetry denotes a red blood cell population containing an increased proportion of osmotically fragile red blood cells. The mechanical fragility of the red blood cells of the infected monkeys was also greater than that of the normal controls. Inspection of the data on percentages of intracellular parasites given in Table I suggests that the parasitized erythrocytes, especially those containing schizonts, were largely responsible for both the increased osmotic and mechanical fragilities of the infected samples. If this were so, selective destruction of the infected cells by prolonged mechanical trauma should produce 2 effects: (a) Decrease of the percentage of parasitized cells; (b) some tendency for the osmotic fragility curve of the traumatized sample to return toward normal.

---

[2] Geiman, Q. M., Anfinsen, C. B., McKee, R. W., Ormsbee, R. A., and Ball, E. G., *J. Exp. Med.*, 1946, **84**, 583.

Inspection of the data in Table I shows that effects (a) and (b) occurred in the 5 instances in which complete tests of infected blood were made. An example of effect (b) is shown in curves 3 and 4 in Fig. 1 (Exp. 45-57). It is to be noted that mechanical trauma usually was especially effective in destroying red blood cells containing schizonts. The negligible effect of trauma on the osmotic fragility of normal monkey blood is shown by the similarity of curves 1 and 2 and by the average values in Table I.

Discussion. Because in some instances the plasmas of the infected monkeys contained hemoglobin obvious to the naked eye, and in one animal hemoglobinuria was observed, the increased osmotic and mechanical fragilities of the parasitized red blood cells are considered to have a possible relationship to the mechanism of the production of the anemia in human malaria and possibly to blackwater fever. Theoretically, the parasites could increase the mechanical fragility of the infected red blood cells by damaging the cell membranes, by increasing cohesion between the cells,[3–5] or by increasing their volume and consequently their spheroidicity.[1] Such increased spheroidicity, measurable as increased osmotic fragility, could be brought about by damage to the cell membrane sufficient to affect its permeability, by the expanding growth of the parasites or by increase of the total osmotic pressure of the cell due to the metabolic activities of the parasites within. Tests for the presence of agglutinins for red blood cells at room and ice box temperatures in the sera of 2 infected monkeys were negative.

The motion of the circulation could very well, because of the increased mechanical fragility of the red blood cells,[6,7] play a part in determining the time of release of the mature parasites and in limiting the percentage of parasitized cells in active circulation. Areas of less active vascular flow might thus provide conditions favorable for the maturation of large numbers of parasites. Osmotic lysis there or the sudden reentry of such unusually mechanically fragile cells into active circulation might contribute in man to the hemolytic crisis of blackwater fever.

Summary. Experiments with the blood of control and of *Macaca mulatta* monkeys infected with *Plasmodium knowlesi* indicate that the parasitized red blood cells develop increased osmotic fragility and *in vitro* are selectively destroyed by mechanical trauma. Consequently, it is suggested that because of this increased mechanical fragility of the red blood cells the motion of the circulation *in vivo* plays a part in the production of the hemolytic anemia in malaria of monkeys and, by analogy, of man.

---

[3] Dameshek, W., and Miller, E. B., *Arch. Int. Med.*, 1943, **72**, 1.

[4] Stats, D., Proc. Soc. Exp. Biol. and Med., 1943, **54**, 305.

[5] Lee, J. S., Puh, Y. C., and Tsai, C., *Proc. Chinese Physiol. Soc., Chengtu Branch*, 1944, **2**, 59.

[6] Meltzer, S. J., and Welch, W. H., *J. Physiol.*, 1884, **5**, 255.

[7] Rous, P., and Turner, J. R., *J. Exp. Med.*, 1916, **23**, 219.

15622

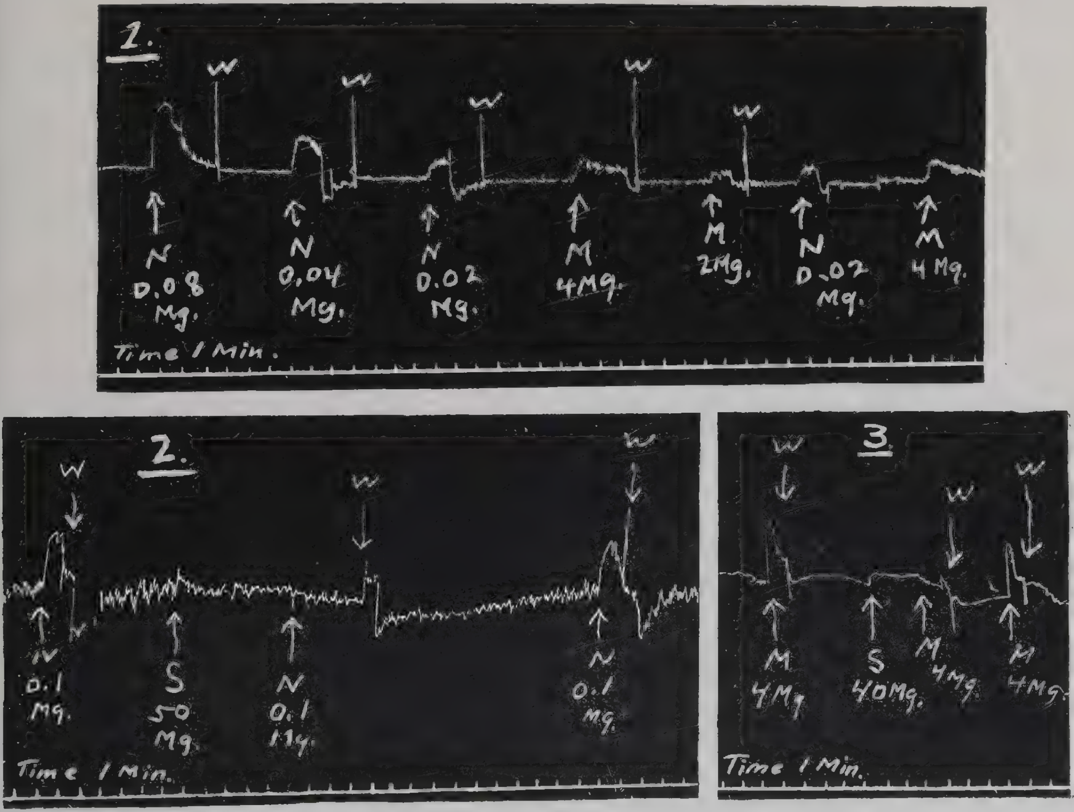
Some Comparative Observations on *l*-Nicotine and Myosmine.

Anthony M. Ambrose and Floyd DeEds.

From the Pharmacology Division, Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, U. S. Department of Agriculture, Albany, Calif.

Myosmine* differs structurally from *l*-nicotine* by lacking the methyl group on the nitrogen of the pyrrolidine ring, which has been converted to a pyrroline ring by a double bond on the 2 carbons adjacent to the pyridine ring. The present report summarizes data on the acute toxicity of these 2 alkaloids for albino rats, and compares their actions on the isolated intestinal and uterine strips of guinea pigs.

The acute toxicity of myosmine was determined orally in 35 rats, and intraperitoneally in 12. The same numbers of rats and the same routes of administration were used for the determination of toxicity of *l*-nicotine. Both alkaloids were dissolved in distilled water and the pH of the solutions



Effect of *l*-Nicotine and Myosmine on the Isolated Guinea Pig Intestine.

Notations in all 3 figures are the same: N—nicotine; M—myosmine; S—sodium sulfathiazole; W—wash. Time intervals, 1 minute.

Fig. 1. Comparative effect of *l*-nicotine and myosmine. *l*-Nicotine appears to be *ca.* 200 times more effective than myosmine. (0.02 mg *l*-nicotine $\rightleftharpoons$ 4 mg myosmine).

Fig. 2 and 3. Showing the inhibiting effect of sulfathiazole on nicotine and myosmine contraction.

---

* The samples of myosmine and *l*-nicotine were prepared by Dr. Abner Eisner of the Eastern Regional Research Laboratory.

adjusted to approximately 7 with concentrated hydrochloric acid.

The approximate oral LD_{50}, expressed as mg per kg of body weight, for myosmine and *l*-nicotine was 1875 and 188 respectively. After intraperitoneal administration the approximate LD_{50} for myosmine and *l*-nicotine was 190 and 30 respectively. The symptoms of toxicity produced by the 2 alkaloids were essentially the same, although the convulsions produced by large doses of myosmine were somewhat less violent than those produced by *l*-nicotine.

Segments of either intestine or uterus were suspended in a 50 cc bath of Ringer-Locke solution at 38°C and connected to a frontal writing lever for kymographic recording. In each case the effect of *l*-nicotine was first determined before myosmine was added.

Myosmine in concentration of 4 mg/50 ml (1:12,500) caused contraction of the isolated intestinal segments similar to that produced by *l*-nicotine in concentrations of 0.02 mg/50 ml (1:2,500,000). On the isolated uterus no effect was produced by either myosmine or *l*-nicotine.

Recently Pick, Brooks and Unna[1] have reported on the inhibitory effect of sulfathiazole on the activity of *l*-nicotine on the intestine. We have confirmed their studies and have extended this procedure to include myosmine.

Like nicotine, the effect of myosmine was inhibited by suitable doses of sulfathiazole. Removal of the sulfathiazole from the bath and washing the segments with fresh Ringer-Locke solution promptly restored the sensitivity of the intestinal segment to both myosmine and *l*-nicotine. The results are illustrated in Fig. 1-3. A detailed description of the individual tracings is given under the figures.

Summary. 1. The acute toxicity of myosmine was about one-tenth that of *l*-nicotine after oral administration. Intraperitoneally myosmine was about one-sixth as toxic as *l*-nicotine. 2. On isolated intestinal strips of guinea pigs, contraction by myosmine required about 200 times the concentration necessary in the case of *l*-nicotine. 3. Sulfathiazole inhibited the action of both myosmine and *l*-nicotine on isolated intestinal strips.

---

[1] Pick, E. P., Brooks, G. W., and Unna, K., *J. Pharm. and Exp. Therap.*, 1944, **81**, 133.

15623

Effects of Colchicine on the Frog in Relation to Ovulation and Early Development.

G. THOMAS SAMARTINO AND ROBERTS RUGH.

From the Department of Biology, Washington Square College, New York University.

The property of inhibiting mitosis and inducing polyploidy[1-3] has made of colchicine a valuable tool in biological research. It has been used in studies on the rate of mitosis in various tissues.[4-6] It has been demonstrated that colchicine prevents the rise in viscosity[7-9] in the cell which normally occurs at fertilization and cell division and also lowers the surface tension in the dividing cell.[10] It has been used in studies on malignant cells.[11–14] The series of experiments herein reported were done with the purpose of seeking explanations for various phenomena that occur during amphibian ovulation, fertilization and early development.

---

[1] Blakeslee, A. F., and Avery, A. G., *J. Heredity*, 1937, **28**, 393.

[2] Kostoff, O., *Nature*, 1938, **142**, 753.

[3] Maba, F., *Jap. J. Genetics*, 1939, **15**, 344.

[4] Brues, A. M., *J. Physiol.*, 1936, **86**, 63.

[5] Allen, E., and Credick, R. N., *Anat. Rec.*, 1937, **69**, 191.

[6] Buschke, W., Friedenwald, J. S., and Fleishman, W., *Bull. Johns Hopkins Hosp.*, 1943, **73**, 143.

[7] Beams, H. W., and Evans, T. C., *Biol. Bull.*, 1939, **77**, 328; 1940, **79**, 188.

[8] Nebel, B. R., and Ruttle, M. L., *J. Heredity*, 1938, **29**, 3.

[9] Wilbur, K. M., PROC. SOC. EXP. BIOL. AND MED., 1940, **45**, 696.

[10] Wada, B., *Cytologia*, 1940, **11**, 96.

Materials and Methods. The experiments were of 2 kinds: *in vivo* and *in vitro*. In the *in vivo* experiments, adult, preovulatory female and adult male *Rana pipiens* were injected in the abdominal cavity for a number of days with colchicine in solution. After several days the females were injected with an excessive dose of pituitary glands in order to induce them to ovulate, and the eggs fertilized with a sperm suspension of minced testes.[15] In the experimental males the left testis was fixed for histological examination and the right was used to fertilize normal eggs. The motility and virility of spermatozoa, as used in suspensions for fertilization, were also tested. The *in vitro* experiments consisted of exposing eggs of *Rana pipiens*, at various stages of development, to varied concentrations of colchicine at different temperatures, but keeping a constant volume of 2 ml of solution per egg or larva.

Experimental Results. The physiological and toxic reactions of the adult frogs to the drug were in accordance with what is known.[16] A pink fluid was found in the subcutaneous lymph spaces. There was a diffuse congestion and inflammation of the mucous membranes of the abdominal viscera. There were numerous small petechiae on the surfaces of the muscles of the limbs, under the skin. There was a blanching of the kidney and adrenal. The gastro-intestinal mucosa was markedly inflamed and the intestines were distended with gas and a frothy mucus. The animals became sluggish, lost the corneal reflex, their proprioceptive senses, and showed a lack of limb coordination. With certain doses of colchicine (varying greatly in different frogs: 0.05 mg, 0.1 mg, 0.2 mg, 0.5 mg, 1.0 mg, and 2.0 mg O.D.) ovulation could be suppressed. In some cases a few eggs were shed (less than a hundred), but these could not be fertilized by the usual technic.[15] In the injected males there was no effect upon the sperm, nor did the histological picture of the testes change with a dosage of 0.05 mg O.D. However, *in vitro* it was found that sperm in concentrations of colchicine from 1:1.000 to 1:70,000 retained their motility (for at least 1½ hours), but lost their ability to activate the eggs. None of the eggs so exposed to spermatozoa ever developed, though most of them rotated.

In the *in vitro* experiments it was found that the developing egg could not survive stages No. 7, No. 12, No. 14-15 in the more concentrated solutions of colchicine (1:10,000 and stronger); eggs in weaker solutions showed meroblastic cleavage. Eggs in colchicine solutions of 1:50,000 and weaker (up to 1:1,000,000) showed little or no deleterious effects. Eggs placed in colchicine after stage No. 7 continued to develop but died at stage No. 12 as exogastrulae. Eggs placed in colchicine after stage No. 12 went on to stage No. 14-15 where the neural folds never fused, and the larvae showed tendencies toward acephalia and microcephaly. Eggs placed in colchicine after stage No. 16 never seemed to be able to hatch out of the jelly capsule, yet if the capsule were ruptured for them, they went on to develop apparently normally. In cases where the larvae were subjected to colchicine just as an organ anlage was about to start to grow, the anlage was suppressed, and even after the larva was removed from the solution and returned to spring water, the anlage failed to develop, *e.g.* the gill did not develop at all or else it grew markedly stunted and without circulation.

In cases where embryos survived the exposure to the colchicine solution and developed through stages No. 7, No. 12, No. 14 they showed a tendency toward microcephaly,

---

[11] Dustin, A. P., *Bull. Acad. Roy. Med. Belg.*, 1934, **4**, 487.

[12] Lits, F. J., and Kirschbaum, A., *Anat. Rec.*, 1938, **70** (suppl. 3), 52.

[13] Amoroso, E. C., *Nature*, 1935, **135**, 266.

[14] Clearkin, P. A., *J. Path. and Bact.*, 1937, **44**, 469.

[15] Rugh, R., *Experimental Embryology*, New York University Bookstore, 1942, p. 25.

[16] Cushny, A. R., *Pharmacology and Therapeutics*, Lea and Febiger, 1940, p. 686.

and a few appeared to be acephalous. In addition these embryos showed stunting, distortion, wrinkling of the ectoderm, curvatures of the body, poorly developed gills without circulation, and exogastrulation. In all the cases the rate of development of the experimental larva lagged behind that of the control; the greater the concentration of colchicine, the greater the lag. In no instance was the size of the experimental greater than that of its control.

Microscopic examination failed to show any differences in the testes; it showed the involution in meroblastic cleavage to have been normal in mechanism, but that it had occurred at a much higher point of latitude on the egg mass than normally, leaving a large amount of yolk unincorporated, so much so that often hemi-embryos resulted which soon died. Mitotic (metaphase) figures were scarce. However, there was an abundance of apparently resting and pycnotic nuclei. In *Rana pipiens* it was noticed that some of these nuclei were 2 and 3 times the size of a normal nucleus, either in discrete masses or bound together by thin filaments of chromatin material.

Discussion. The presence of the pink fluid in the subcutaneous lymph spaces and the blanching of the kidney and suprarenal body, indicate either a loss of red blood cells with subsequent hemolysis, or hemolysis within the blood vessel and subsequent excretion of free hemoglobin by the kidney, and loss intercellularly in the capillary beds of the lymph spaces. The evidence that colchicine is a capillary poison is evidenced by the marked dilatation of the capillaries. The great inflammation present in the intestines can be accounted for, since this is the chief site of the excretion of the drug. The insensitivity of the animals to mechanical stimuli is probably the result of paralysis of nerve endings, and the depression of sensory and motor centers in the central nervous system.[17]

The mechanisms by which colchicine suppressed ovulation, and destroyed the virility of the sperm but not their motility, are moot questions. Clearly, rotation of the egg cannot be accepted as a criterion for penetration by the sperm, for it seems improbable that of all the eggs observed to rotate (several thousand), not one should have cleaved, even on a pathenogenetic basis.

When one considers that the gross deformities in the embryos resulted from a lack of cell division in some areas, and from unequal or unbalanced growth, it becomes evident that there is a qualitative difference in cells about to undergo differentiation and those that are dividing without differentiation. This is shown by their differential susceptibility not only to colchicine, but also to other chemical agents as well.[18,19]

Opinion is divided on the question of whether colchicine is a mitotic stimulant or inhibitor.[20-22] From our experiments we must conclude that it acts as a depressant in mitosis in the frog. The drug prolongs the mitotic cycle, and in certain concentrations stops it completely. The mechanism can only be speculated upon. Yet, the experimental evidences of many workers tend to support Bernal's theory[23] of negative tactoids in mitosis and spindle formation. The lack of the requisite increases in viscosity, due to the influence of colchicine, may account for the lack of spindle formation[7,24-26] and for the bizarre figures seen in colchicine-treated cells.[20]

It has been stated[27] that, as a result of their reactions to colchicine, anuran and urodelan tissues do not constitute a homogeneous class of tissues. Incidental observations on *Amblystoma punctatum* demonstrated that the number of pycnotic nuclei and metaphase figures were not the same in the frog and salamander. Yet, if one considers the pycnotic nuclei as former metaphases which have become agglutinated, and the pseudo resting stages as transitionals from the colchicine metaphase figure to the pycnotic nucleus, then the sum total of all 3, in each species, is about equal. Then, the reaction to colchicine is qualitatively and quantitatively the same in both the frog and the salamander, both microscopically and grossly.

---

[17] Jacobi, K., *Arch. f. exp. Path. und Pharm.*, 1890, **27**, 119.

[18] Waterman, A. T., *Biol. Bull.*, 1939, **76**, 162; 1940, **78**, 29.

[19] Brues, A. M., and Cohen, A., *Biochem. J.*, 1936, **30**, 1363.

[20] Laur, C. M., *Ann. Anat. Path. Med.-Chir.*, 1938, **15**, 792.

[21] Paff, G. H., *Am. J. Anat.*, 1939, **64**, 331.

[22] Bureau, V., and Vilter, V., *C. R. Soc. Biol.*, 1939, **132**, 553.

[23] Bernal, J. D., *Cell and Protoplasm*, 1940, p. 199.

[24] Brues, A. M., and Jackson, E. B., *Am. J. Cancer*, 1937, **30**, 504.

[25] Ludford, R. J., *Arch. f. exp. Zellforsch.*, 1936, **185**, 411.

[26] Barber, H. N., and Callan, H. G., *Proc. Roy. Soc. (B)*, 1943, **131**, 258.

[27] Mills, K. O., *J. Morph.*, 1939, **64**, 89.

Summary and Conclusions. Colchicine was used in concentrations ranging from 1:1000 to 1:1,000,000. It was found to act as a capillary poison, to prevent ovulation when injected into sexually-mature female frogs prior to anterior pituitary stimulation, to prevent cleavage of normal eggs exposed to spermatozoa previously treated with colchicine, to affect gastrulation to such an extent that exo-gastrulae were the rule, to inhibit the appearance of organ anlage when applied just before the normal appearance time, to retard general growth of the larvae to such an extent that cephalic structures were markedly stunted and hatching prevented. The cytological picture was one of many pycnotic nuclei, occasional multinucleated cells, few metaphase figures, and apparent resting stages of mitosis.

15624

Simultaneous Cultivation of Typhus Rickettsiae and Influenza Virus in the Developing Chick Embryo.

Laurella McClelland. (Introduced by R. D. Defries.)
From the Connaught Medical Research Laboratories, University of Toronto.

Over the past few years, large numbers of developing chick embryos have been used in this laboratory for the production of vaccines prepared from typhus fever rickettsiae and from influenza virus. In the preparation of typhus vaccine, the rickettsiae are cultivated in, and harvested from the yolk sac, whereas for influenza vaccine, the influenza virus is harvested from the allantoic fluid. In each instance, the remainder of the egg-contents is discarded. Thus, the allantoic fluid of the typhus eggs and the yolk sacs of the influenza eggs are wasted in the preparation of typhus vaccine and influenza vaccine, respectively. It had been observed, however, that in eggs inoculated with typhus rickettsiae in dilutions that did not result in the early death of the embryo, very few rickettsiae were found in the allantoic membranes. Moreover, it did not seem likely that rickettsiae, even if present, would be adsorbed on and subsequently eluted from fowl red blood cells, after the manner of the influenza virus.[1] Conversely, the adapted form of influenza virus used as seed material for vaccine production does not proliferate to any extent in the yolk sac.[2] In each instance, then, the portion of the egg that is discarded could be salvaged and used to advantage if both agents could be grown simultaneously in the same egg. The present communication records the results of a series of experiments in which vaccines were prepared from eggs inoculated with both typhus rickettsiae and influenza virus.

In preliminary experiments, it was found that an egg previously infected with typhus could actually be reinfected with influenza. In order to obtain a good yield of influenza, it would be essential, however, to regulate very carefully the dose of typhus. Accordingly, from the results of egg titrations[3] carried out on each batch of typhus seed material, a dose was selected that would produce an infection sufficiently heavy to kill most of the embryos on the 7th, 8th and 9th day following inoculation. Thus, the typhus-infected eggs could be reinoculated with influenza virus on the 5th day following the original rickettsial inoculation, and so allow the proliferation of the influenza virus for 2, 3 or 4 days before the death of the embryo.

---

[1] Hirst, G. K., *J. Exp. Med.*, 1942, **75**, 195.

[2] Beveridge, W. I. B., Burnet, F. M., and Williams, S. E., *Australian J. Exp. Biol. and Med. Sc.*, 1944, **22**, 1.

Materials and Methods. Experiments were planned according to the following schedule.

A. Double infection of developing chick embryos with:

1. Epidemic strain of typhus rickettsiae and influenza virus A.
2. Epidemic strain of typhus rickettsiae and influenza virus B.
3. Murine strain of typhus rickettsiae and influenza virus A.
4. Murine strain of typhus rickettsiae and influenza virus B.

B. Vaccine preparation from eggs infected with both typhus and influenza:

1. Typhus vaccines from yolk sacs.
2. Influenza vaccines from allantoic fluid.

Eggs. White Leghorn eggs that had been incubated for 7 days were supplied by a dealer.

Seed Materials. All seed materials were derived from the stocks used in routine vaccine production.

For rickettsial inoculations, the Breinl strain of epidemic typhus and the Castaneda strain of endemic (murine) typhus were employed. For influenza virus inoculations, the PR8 strain of influenza A and the Lee strain of influenza B were used.

Inoculations and Incubation. Eggs that had been incubated for 7 days were candled in the upright position and the lower borders of the vascular membrane were indicated on the shell. The typhus rickettsiae were inoculated directly into yolk from the side of the egg and below the vascular area. After 5 days' further incubation, at 35°C, the eggs were candled and the dead embryos were discarded. Influenza virus was then introduced into the allantoic space of the surviving eggs, after which they were returned to the incubator, at 35°C. Thereafter, they were candled twice daily, and the dead embryos were saved for collection of yolk sacs and allantoic fluid.

Harvesting. During the course of the experiments, eggs were harvested and examined individually. The clear allantoic fluid was first aspirated and stored, and a small sample was kept for testing. At time of harvesting, the presence or absence of influenza virus in each sample was determined by examining for its ability to agglutinate fowl red blood cells.[4,5] The yolk sac was then separated, stripped free of yolk and placed in a vial. A small portion of each yolk sac membrane was used to make an impression smear that was stained with methyl violet and examined for the presence of rickettsiae. Allantoic fluids were stored in vials in the refrigerator at 4°C; yolk sacs were stored in vials in a CO_2 ice-box.

Preparation of Influenza Vaccine. Following the individual harvesting of each egg and examination of the samples, the allantoic fluids were combined so that fluids from eggs harvested on the same day were pooled together. Hemagglutination titers[6] were determined for each pool. After this estimation, all the pools were combined and were used for the preparation of a vaccine.[7] The vaccine was prepared by adsorbing the virus on fowl red blood cells in the cold, separating the supernatant fluid from the agglutinated cell mass and eluting the virus from the latter into one-tenth volume of saline at 37°C. To each vaccine was added a final concentration of 0.05% formalin and 0.01% merthiolate.

---

[3] Craigie, J., McClelland, L., and Parker, R. C., Memorandum No. 5 (Project Med. 8). Submitted to the Assoc. Comm. Med. Research, Nat. Research Council Can., May, 1943. (Withheld from publication for security reasons.)

[4] Hirst, G. K., *Science*, 1941, **94**, 22.

[5] McClelland, L., and Hare, R., *Canad. Pub. Health J.*, 1941, **32**, 530.

[6] Hirst, G. K., *J. Exp. Med.*, 1942, **75**, 49.

[7] Francis, T., and Salk, J. E., *Science*, 1942, **96**, 499.

TABLE I.
Protocol of a Typical Experiment.

Exp. No. 3 No. of eggs: 24	Date: Aug. 30, ’45	Temp. of incubator: 35°C

Age of embryos at time of inoculation with rickettsiæ: 7 days
Strain and dose of rickettsiæ: Epidemic, Breinl (56E. 27.3.45); 0.2 ml of a 2×10^{-5} dilution of seed yolk sac per egg
Site of inoculation: side of egg, below vascular area, into yolk
Survivors after 5 days: 20 eggs (4 discarded)

Age of embryos at time of inoculation with influenza virus: 12 days
Strain and dose of influenza virus: B, Lee (Francis) E_3; 0.2 ml of a 10^{-2} dilution of seed allantoic fluid per egg
Site of inoculation: side of egg, into allantoic space.

TABLE II.
Results of Inoculations of Developing Chick Embryos with Typhus Rickettsiæ and Influenza Virus.*

Days after inoculation					
With typhus	With influenza	No. of dead eggs	Egg No.	Agglutination with fowl red blood cells (for influenza)	Yolk sac smear (for typhus)
7	2	7	1	positive	+++
			2	”	++
			3	”	++++
			4	”	+++
			5	”	+++
			6	”	+++
			7	contaminated	
8	3	6	8	positive	+++
			9	no fluid obt’d	++++
			10	positive	+++
			11	”	++++
			12	”	+++
			13	”	++++
9	4	7	14	”	++++
			15	”	+++
			16	”	++++
			17	”	++++
			18	”	+++
			19	”	+++
			20	”	+++

Hemagglutination titer of allantoic fluids:
(a) pooled on 2nd day after inoculation with influenza: 1/192
(b) pooled on 3rd day after inoculation with influenza: 1/256
(c) pooled on 4th day after inoculation with influenza: 1/192
Hemagglutination titer of finished influenza vaccine: 1/2000

* See Table I.

Preparation of Typhus Vaccine. The frozen yolk sacs were allowed to thaw, were pooled and were fragmented by shaking with glass beads. Saline containing 0.75% formalin was added to the pooled yolk sacs to make a 15% suspension by weight and to kill the rickettsiae. After 3 days at 4°C, the yolk sac emulsion was processed by treatment with ethyl ether according to the method[3] used in this laboratory for the preparation of typhus vaccine.

Experiments and Results. The protocol of a typical experiment is shown in Table I. In Table II are shown the results of the inoculations.

Testing for Antigenicity. Four influenza and 4 typhus vaccines were submitted for antigenic testing. Determinations of antigenicity were carried out by those* who were responsible for the routine antigenicity testing in the influenza and typhus vaccine units.

The 4 influenza vaccines tested (2 influenza A vaccines and 2 influenza B vaccines) were antigenic in mice and passed the requirements established by the U. S. Public Health Service, Division of Biologics Control as used in this laboratory in the antigenic testing of influenza vaccines.[8]

The 4 typhus vaccines tested (2 epidemic typhus vaccines and 2 murine typhus vaccines) were antigenic in mice and protected them against challenge doses of the toxic material regularly employed in this laboratory for the antigenic testing of typhus vaccines.[9]

Testing for Cross Immunity. Half of the mice that had been inoculated with influenza A vaccine and that had survived the challenge dose of influenza A virus were challenged, after a suitable interval, with epidemic typhus; the other half were challenged with murine typhus. This manner of cross challenging was repeated with each group of vaccinated mice that survived its own particular challenge material. Minimal doses of the heterologous challenge material were administered in each instance in order to detect any evidence of cross protection.

The groups of mice that had been protected with influenza vaccine, and that had survived the challenging dose of influenza virus, showed no evidence of immunity to either the epidemic or the murine strain of typhus rickettsiae.

In the groups receiving typhus vaccine, most of the mice that had survived the challenging dose of typhus, died following a challenge with influenza virus. The few surviving mice were sacrificed on the 10th day after the administration of influenza virus. All showed evidence of having been infected with influenza, as typified by lung consolidation ranging from 1 to 4+.

Summary and Conclusions. (1) Developing chick embryos were inoculated with rickettsiae of typhus fever, following which the same eggs were reinoculated with the virus of influenza. Two strains of rickettsiae were employed, namely, the Breinl strain of epidemic typhus and the Castaneda strain of murine (endemic) typhus. Also, 2 strains of influenza virus were employed, namely, the PR8 strain of influenza A and the Lee strain of influenza B. The groups of developing chick embryos inoculated with typhus and influenza, included the 4 possible combinations of rickettsiae and virus, *viz.* epidemic typhus and influenza A, epidemic typhus and influenza B, murine typhus and influenza A, and murine typhus and influenza B.

(2) Rickettsiae, inoculated into the yolk sac, were recovered from the yolk sac membranes, and typhus vaccines were prepared therefrom.

(3) Influenza virus, inoculated into the allantoic cavity, was recovered from the allantoic fluid and influenza vaccines were prepared therefrom.

(4) All the vaccines passed the requirements established for typhus and influenza vaccines produced in this laboratory.

(5) There was no evidence of immunity to influenza in the mice inoculated with typhus vaccine, and conversely, there was no evidence of immunity to typhus in the mice receiving immunizing doses of influenza vaccine.

---

* I gratefully acknowledge the cooperation of Mrs. Dorothy M. Mackenzie and Dr. John F. Crawley and the departments in which they work. Mrs. Mackenzie was responsible for the antigenic testing of the influenza vaccines. Dr. Crawley was responsible for the antigenic testing of the typhus vaccines and for the cross challenging of the influenza mice with typhus.

[8] Hare, R., and Mackenzie, D. M., in preparation.

[9] Craigie, J., in preparation.

15625

An Improved Feeder for Mice.

H. P. RUSCH, V. R. POTTER, AND J. A. MILLER.

From the McArdle Memorial Laboratory, Medical School, University of Wisconsin, Madison, Wisc.

In studies which require an accurate determination of food intake the spilling of food and its contamination by excreta must be kept to a minimum. Mice are notorious for their perversity in these respects. Feeders constructed along the general pattern given in the accompanying diagrams have been in use in our laboratory for over a year and have given very satisfactory service in measurements of the average caloric intakes of groups of 2 to 24 young adult mice receiving synthetic diets of widely different compositions. The feeder consists of 2 easily assembled parts. The upper piece contains compartments each of which accommodates only one mouse at a time and which are floored with ¼ inch square wire mesh. The wire mesh extends on each side of this assembly so that it may be slipped under the ledges of the food tray from either end. Thus assembled the top piece rests freely on the food in the tray and descends into the tray as food is consumed. The upper piece is readily removed when additional diet is added to the food tray. The feeders may be constructed from No. 26 galvanized iron sheeting and the joints soldered or spot-welded; the shop on this campus employed a jig for ease of manufacture. Details of a feeder for mice weighing between 15-30 g are shown in the accompanying diagram.

The small compartments, the wire mesh, and the ledges prevent the food from being spilled or contaminated. The average daily spillage of food for 10 adult mice fed *ad libitum* from a 10-compartment feeder

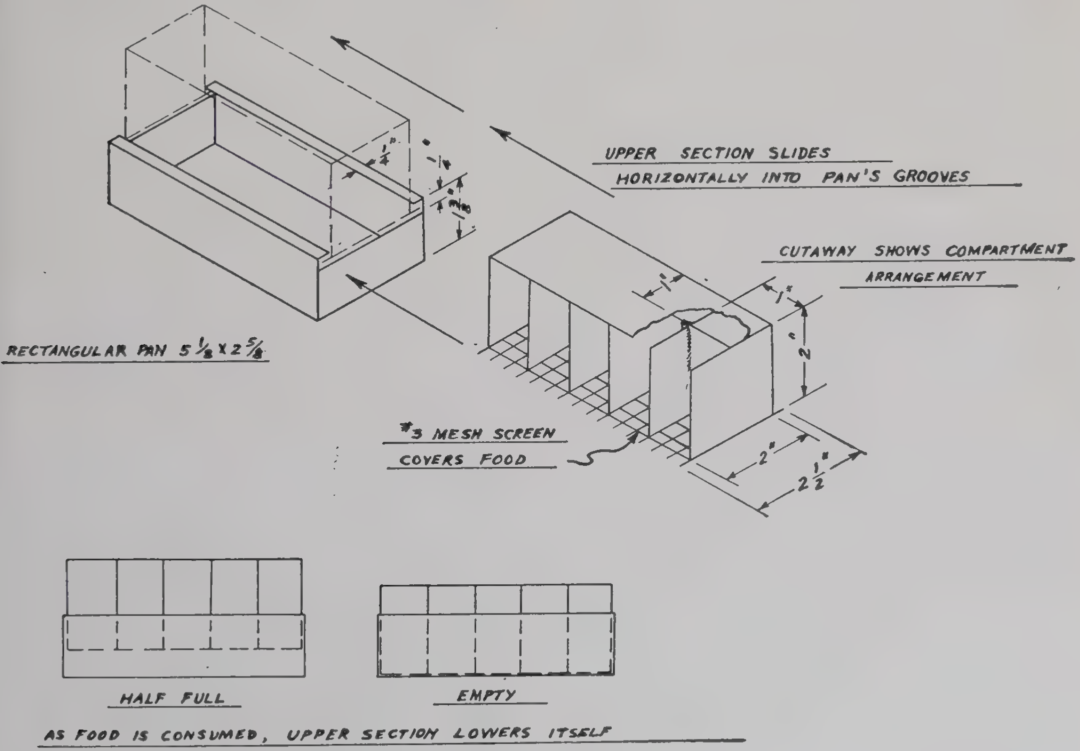

UPPER SECTION SLIDES HORIZONTALLY INTO PAN'S GROOVES
CUTAWAY SHOWS COMPARTMENT ARRANGEMENT
RECTANGULAR PAN 5 1/8 x 2 5/8
1/4"
1/8"
3/8"
1"
1"
2"
2"
2 1/2"
#3 MESH SCREEN COVERS FOOD
HALF FULL
EMPTY
AS FOOD IS CONSUMED, UPPER SECTION LOWERS ITSELF

FIG. 1.

amounts to only 0.1 g or less. In feeding diets *ad libitum* we employ weighed amounts of food about 10% in excess of the expected consumption. The feeders may be constructed with varying numbers of compartments but when the food intake is being restricted below the *ad libitum* level, they should at least equal the number of mice in the cage. The compartment feature gives reasonable assurance that all of the mice in a group have an equal chance to feed when the food intake is restricted. In this regard, the practice[1] of dividing the animals in any group into smaller groups of narrower weight ranges is useful. To prevent spillage and contamination of the food with excreta the compartments must allow the entry of only the fore part of the animal; they should not accommodate the whole animal. The use of similar feeders, with compartments and wire mesh of the appropriate size, would appear entirely feasible for weanling mice or for rats of varying size.

---

[1] Tannenbaum, A., *Cancer Research*, 1945, **5**, 609.

15626

The Effect of Exercise on Mortality of Animals Poisoned with Diphosgene.

S. Postel, J. M. Tobias, H. M. Patt, and R. W. Gerard.
*From the Department of Physiology, The University of Chicago.**

Absolute rest for victims of pulmonary irritants has until recently been the rule of thumb. The clinical consensus against exercise is clearly represented in the directions for first aid treatment of lung irritant casualties given in a U. S. War Department Field Manual dated September 7, 1942:[1]

"Patients suspected of having breathed lung irritant gas should be given absolute rest. All casualties known or suspected of having been seriously exposed should be considered as litter cases . . . when he has been carried to a place free of gas, the gas mask is removed, his clothing loosened and the man kept completely still, relaxed and warm with blankets."

Strict adherence to these regulations precludes any attempt by the gassed soldier to make his way to an aid station during the interval between exposure and the appearance of pulmonary edema. The present study was designed to test the validity of prohibiting exercise during the clinical latent period. Similar work has been done independently and at about the same time by I. de Burgh Daly and co-workers, who found that exercise on an endless carpet had no injurious effect on rats[2] and dogs[3] after exposure to L Ct-50 concentrations of diphosgene or on dogs gassed with phosgene.

Preliminary Experiments. Swimming was used as a controllable and relatively constant form of vigorous exercise requiring virtually no apparatus for mice, rats or dogs. Its disadvantage, temporary change to a water environment, was controlled by experiments testing the effects of wetting and temperature changes immediately after gassing.

While normal mice tolerated prolonged wetting (standing in a shallow pan of water for 42 hours) with water and air at 29°C, but not at 17°C, gassed mice did poorly under prolonged wet conditions both at 28°C and at 15°C (Table I). There are no figures for "dry cold" survival of normal mice but

---

* This work was done as part of a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the University of Chicago.

[1] *Defence Against Chemical Attack*, U. S. Government Printing Office, Washington, D.C., September 7, 1942, p. 28.

[2] Daly, I. de Burgh, and Ritchie, A. O., personal communication.

[3] Daly, I. de Burgh, Fegler, J., and Ritchie, A. O., personal communication.

TABLE I.
Influence of Wetting and Temperature on Normal and Diphosgene Gassed Mice.

Gas exposure Mg/L.	Gas exposure Min.	Condition* (temp. °C)	No. of mice	% alive at 36 hr	X^2	P
Normal controls		42 hr wet at 29°C	20	100	10.80	$< .01$
		42 " " " 17	20	50		
0.45	10	42 " dry " 28	25	70	9.80	$< .01$
		42 " " " 15	25	20		
		42 " wet " 28	25	0		
		42 " " " 15	25	0		
0.43	10	1 " dry " 30	20	20	0.322	>7.50
		1 " " " 14	20	30		
		1 " wet " 30	20	25		
		1 " " " 14	20	15		

* Low temperatures (14°-17°C) maintained in constant temperature room.

TABLE II.
Influence of Swimming on % Survival of Gassed Mice (7 experiments, 420 animals).

	% surviving at 36 hr						
Gas conc'n (mg/L):	0.33	0.40	0.42	0.42	0.46	0.46	0.49
Dry control	70	45	30	40	20	15	0
Shallow water control	45	65	—	20	—	20	0
Moderate early swim	65	70	50	60	35	—	10
Heavy early swim	30	65	—	—	—	10	—
Swim at 1.5 hr	—	—	—	15	—	—	—
Swim at 3.0 hr	—	—	—	20	—	5	—
X^2	6.77	3.22	1.66	9.13	1.14	1.07	4.17
X^2 at $P = 5\%$	7.81	7.81	3.84	9.49	3.84	7.81	5.99

TABLE III.
Pooled % Survival of Gassed Mice in 5 Treatment Classes.

Treatment class	No. of groups	No. of mice	Mean % survival
Dry controls	7	135	31.4
Shallow water controls	5	79	30.0
Moderate early swim	6	115	48.3
Heavy early swim	3	53	35.0
Late exercise	3	48	13.3

$$F \frac{\text{between means}}{\text{within classes}} = 1.27;\ n_1 = 4,\ n_2 = 19$$

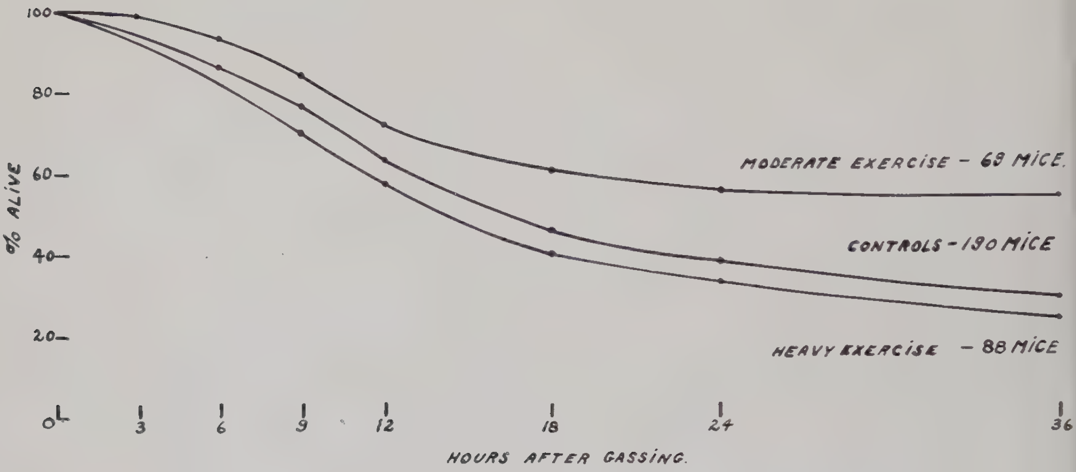
$$F \text{ at } 5\% = 2.89$$

gassed mice kept dry at 15° showed a mortality more than twice as great as a comparable group at 28°C. Reducing the exposure to wet and cold to one hour, beginning half an hour after gassing, eliminated their effects; mortality of gassed mice then being the same under dry cold, wet cold, dry warm or wet warm conditions (Table I). Experiments were therefore planned with a swimming period of not longer than an hour at a water temperature between 25°C and 30°C and with the animals dried afterwards. Floats were placed in the water from time to time to give mice and rats short rests. All diphosgene exposures for mice were within a range of 0.33 to 0.49 mg/L for 10 minutes, resulting in a mortality of from 25 to 80% at 36 hours.

Results of Exercise Experiments. A. Mice. Four hundred and twenty mice were exposed to diphosgene in 7 experiments. In each, the mice were divided into a dry control

FIG. 1.

SURVIVAL OF MICE EXERCISED AFTER GASSING.



group of from 10 to 25 mice and one or more (up to 5) experimental groups containing 9 to 25 animals. In 5 of the experiments a second control group was kept standing in a pan of shallow water during the swimming period of exercising groups. The results of these experiments, expressed as per cent surviving at 36 hours for the individual groups, are shown in Tables II and III and Fig. 1. Moderate exercise consisted of a 20- to 40-minute swim with one or two 5-minute rests; heavy exercise, a one-hour swim with 3 evenly spaced 5-minute rests. This latter taxed animals to the limit; without rest periods, they would have drowned. Exercise was begun within the first half hour after the end of gassing except, as indicated in the last 2 rows of Table II, when 40- to 60-minute swims were begun at 1½ and 3 hours after gassing to test the effect of later exertion.

It is apparent from Table II that the populations of each of the 7 experiments are homogeneous. Differences between the mean survival percentages of the several treatment classes (Table III) are also not significant, as shown by analysis of variance. There is, however, a significant difference between the mean of moderate early and late exercise classes considered apart from the other treatment classes ($F = 6.47$; $n_1 = 1$; $n_2 = 7$). This difference is due either to the inconclusiveness of the 5% point in statistical analysis or to inadequate sampling. If the latter, more experiments might conclusively demonstrate a beneficial effect of moderate, early, postgassing exercise (Table II and Fig. 1) or a deleterious effect of late exercise, or both. In any event, heavy or moderate early exercise, as defined in these experiments, was noninjurious to mice gassed at the levels indicated.

TABLE IV.
Influence of Swimming on Gassed Dog Survival Time.

Treatment	No.	Mean survival time (hr)	$\frac{\sigma}{\sqrt{n}}$
A. Controls	15	14.1	2.31
B. Moderate swim	6	18.0	4.41
C. Heavy swim	8	9.9	1.17

Significance of Differences Between Means

Groups	Observed t	t at 5% P
AB	0.78	2.09
AC	1.62	2.08
BC	1.77	2.18

B. Rats. Forty rats were exposed to a 0.52 mg/L concentration of diphosgene for 10 minutes. Twenty were dry controls and 20 swam immediately after gassing, for 20

TABLE V.
Terminal Lung-weight Body-weight Ratios of Control and Exercised Gassed Dogs.

Treatment	No.	Mean ratio lung wt/body wt %	Mean survival time (hr)	Mean ratio / Mean survival
A. Controls	15	3.82	14.1	2.71
B. Moderate swim	5	4.42	15.6	2.83
C. Heavy swim	8	4.80	9.9	4.84

Significance of Differences Between Mean Ratios.

Groups	Observed F	F at 5%
ABC($N_1 = 2$, $N_2 = 25$	3.06	3.38
AC($N_1 = 1$, $N_2 = 20$	5.88	4.35
AB($N_1 = 1$, $N_2 = 18$	1.72	4.41

minutes with rests. Control mortality was 80%; swimmer mortality 65%. The 15% difference is statistically not significant ($X^2 = 0.502$), but again is in the direction of the beneficial effect suggested above.

C. Dogs. 1. Survival Time. Thirty dogs were exposed to 0.52 to 0.69 mg/L concentrations of diphosgene for 30 minutes, in groups of 6. Three control dogs from each of the 5 groups were wetted during the swimming periods of the exercise dogs. Moderate exercise was a 20-minute swim; severe exercise lasted 40 minutes. One 40-minute swimmer was completely overcome and 2 of the 20-minute swimmers were so exhausted as to be more properly classed with heavy exercises, leaving overall totals of 15 controls, 6 moderately exercised and 8 severely exercised animals. All but 4 dogs died within 30 hours after gassing. Of the 4 exceptions, one lived indefinitely and the other 3 from 43 to 105 hours. In the calculation of mean survival times these 4 dogs were treated as 30-hour survivors, deaths after about 30 hours usually being due to secondary causes.

The mean survival times of the 3 groups are shown in Table IV. The absence of a statistically significant difference between any 2 means indicates the indifferent influence of exercise on survival time.

2. Degree of Pulmonary Edema at Death. Estimates of the extent of pulmonary edema at death on the basis of lung-weight body-weight ratios, were homogeneously distributed, considering the whole dog population (Table V). There was, however, a statistically significant difference between the mean ratios of the controls and severely exercised animals considered alone; none between controls and moderate swimmers or between heavy and moderate swimmers. Considering average rate of lung water deposition (lung-weight body-weight ratio divided by survival time) suggests that the greater terminal ratio of moderate exercisers as opposed to controls, if shown to be significant, would probably be due to a longer mean survival time. Severe exercise, on the other hand, definitely favors increased water deposition. The latter may result from inability of the dogs to absorb whatever water they may aspirate during the swimming period, but one would rather suspect actual aggravation of the edema.

Conclusions. It is clear that the worst that can be said of swimming shortly after diphosgene gassing at mid- or somewhat above mid-lethal doses is that it had no influence on final mortality among mice and rats or on survival time in dogs. There was, in fact, some indication, especially from the finding of a significant difference between survival of moderate early and late exercised mice, that moderate early swimming was beneficial or late swimming deleterious or both. It is striking that in all of the pooled data (see tables), and very often in individual experiments, differences noted, though not statistically significant, are consistently in the direction of increased survival for moderate early exercise in all 3 species, and in the direction of increased mortality for moderate or heavy late exercise in mice and heavy early exercise in dogs.

The dog survival times and lung weights

suggest that "moderate" and "heavy" exercise as distinguished in these experiments are physiologically as well as quantitatively, of different orders of magnitude. Similarly, in the mouse experiments there is no indication of any effect, beneficial or deleterious, of heavy early swimming, as opposed to the indicated beneficial influence of moderate exercise.

Swimming seems a justifiable test of the effects of more natural types of exertion. The control experiments on mice show that the indifferent role of swimming or the perhaps beneficial effect of moderate early swimming does not represent an overcompensation of harmful exercise by a protective action of immersion in tepid water. If anything, swimming, as already indicated, may be responsible for the apparent ill effect of heavy "exercise" in dogs judged by the significantly greater pulmonary edema of heavy swimmers as opposed to controls. Furthermore, it may be valid to assume that, from the point of view of muscular, circulatory and respiratory mechanisms, swimming is at least as vigorous an activity as that envisaged for the soldier gassed at equivalent levels; we have here subjected exertion to a perhaps unduly rigorous test.

Finally, it may be pointed out that exercise by a diphosgene patient or other pulmonary irritant patient during the clinical latent period need not be expected *a priori* to be adding insult to injury. The water and blood demands of muscle during exercise could tend to delay the onset of edema. Once edema and attendant anoxaemia set in, however, increased oxygen demand becomes an undesirable feature of exercise. Since the time of onset of edema is variable, depending mainly on severity of exposure, which is in turn a function of more than just gas concentration and exposure duration, no particular time can be set as the limit of a safe period.

15627 P

Inositol Decholesterization in Old Hens.*†

GEORGE R. HERRMANN.

From the Department of Internal Medicine, University of Texas.

Inositol was found by Gavin and McHenry[1] to prevent the development of a special type of biotin fatty liver, but no such lipotropic activity was demonstrated against thiamin fatty livers. Abels *et al.*[2] found that inositol accounted for the lipotropic property of a lipocaic preparation when given to patients with gastrointestinal cancer. Inositol administered preoperatively, reduced the average concentration of fat to 46% of that of almost uniformly fatty infiltrated livers of untreated patients. Shay[3] could demonstrate no reduction in the size of fatty livers in diabetics nor any changes in the blood cholesterol by the daily administration of 1.2 g of inositol. However, Russakoff and Blumberg[4] reported reduction in the blood cholesterol and cure by inositol in doses of 1 g per day, in a patient with a dermatological condition associated with a disturbed fat metabolism.

---

* With the technical assistance of Anna H. Williams, Lucy Prosise, H. Tom Leigh, John Prewett, and George W. Reimer.

† Inositol was generously supplied and the study was, in part, supported by a grant from the Medical Research Department of the Winthrop Chemical Co.

[1] Gavin, G., and McHenry, W. E., *J. Biol. Chem.*, 1941, **139**, 485; 1943, **148**, 25.

[2] Abels, J. C., Kupel, C. W., Pack, C. T., and Rhoads, C. P., PROC. SOC. EXP. BIOL. AND MED., 1943, **54**, 157.

[3] Shay, H., *Am. J. Digest. Dis.*, 1943, **10**, 48.

[4] Blumberg, H., and Russakoff, A. H., unpublished data, quoted in *Ann. Int. Med.*, 1944, **21**, 848.

TABLE I.
Effect of Inositol on Blood and Tissue Lipids.

	Blood cholesterol, mg/100 ml		Phospholipids mg/100 ml	Tissue Cholesterol, mg/100 g					
				Aorta		Heart		Liver	
	Total	Esters		Total	Esters	Total	Esters	Total	Esters
Controls	274.8	166	9.6	230	176.2	275	223	340	240
36 old hens S.D.	±39	±19	±2.7	±41	±20.4	±71	±19	±65	±24
(A) Series (7)									
Inositol 1st Bl.*	254 ±28	201±29	7.5±1.7						
25-30 2nd Bl.	220 ±29	169±26	10 ±1.1						
days 3rd Bl.	202 ±18	151±16	9.4±0.7	210	160	172	126	281	221
				±10	±19	±9.6	±9	±28.5	±23.5
(B) Series (16)									
Ino.† control									
1st Bl.	230 ±29	181±27	8.6±1.3						
Ino. 25 days									
2nd Bl.	206 ±20	160±20	11.3±1.9						
Ino. 55 to 68 days				197	145	167	127	241	178
3rd bl.	176 ±13	140±12	12.9±1.9	±27	±22	±40	±29	±60	±35
(C) Series (23)									
Inositol 1st Bl.	237 ±29	187±31	8.7±1,2						
2nd Bl.	210 ±24	163±20	11 ±1.9						
30-68 days 3rd Bl.	184 ±19	135±16	11.8±1.9	201	155	166	129	253	191
				±38	±24	±37	±29	±55	±93

* Bl.—Bleeding. † Inositol. S.D.—Standard Deviation, ±.

Further studies seemed to be in order and inositol was therefore subjected to animal experimental studies similar to those made with other lipotropic substances.[5]

Two series of old hens from high egg-producing stock were individually caged and given the standard high-fat laying mash (Purina). Blood was withdrawn from 16, and later 7 more, hens for the determination of control levels of total cholesterol, cholesterol esters, total and inorganic phosphorous levels by the methods of Bloor,[6] Bloor and Knudson,[7] and King,[8] respectively.

Inositol, 0.5 g was then added to the diet, and put down the gullet of each hen daily. All the birds were healthy throughout the experiment. After 25 days blood was again removed for the chemical studies and at the sacrifice after 30 days in 7 hens and after 62 days in 16 hens. The aorta and approximately 0.25 g of heart muscle and a similar mass of liver were removed, weighed accurately and macerated with sand and extracted with ether alcohol. The 10 cc extract of each organ was analyzed and the amount of total cholesterol and cholesterol esters in 100 g of each tissue calculated. The statistically-treated data for the 7 hens treated for 25 to 30 days and for the 16 hens treated for 55 to 68 days are set down in Table I and for all 23 inositol-treated hens for comparison with the normals previously established.[5]

It is evident that barely significant reductions in cholesterol in blood and tissues develop in 30 days while definite reductions have been recorded in 55 to 68 days.

Under the conditions of this experiment, the total blood cholesterol dropped about 11% and the cholesterol esters about 13%, but the organic or lipid phosphorous rose about 31% after 25 days of inositol. After 55 to 68 days, the average total blood cholesterol had dropped 22%, the esters 17%, and the phospholipids had risen 50%. The tissue analysis of inositol-treated hens showed reductions in concentration of 9% and 9% of cholesterol total and esters in the aorta in 30 days and 14% and 12% in 52 to 68 days. The heart muscle levels dropped 37.5% and 43% in 30 days and 40% and 41% in 55 to 68 days, and those in the liver were 17.6% and 18.3% in 30 days and 25% and 20% in 55 to 68 days respectively below the levels in the controls established in previous studies.[8]

---

[5] Herrmann, G. R., PROC. SOC. EXP. BIOL. AND MED., 1946, **61**, 229 and 302. (Note: In these two communications the subheading under Tissue Cholesterol total/esters determinations on aorta, heart, and liver were erroneously set down as mg/250 mg instead of mg/100 g to which amounts the calculations had beeen made.)

[6] Bloor, W. R., *J. Biol. Chem.*, 1916, **24**, 227.

[7] Bloor, W. R., and Knudson, A., *J. Biol. Chem.*, 1916, **27**, 107.

[8] King, E. J., *Biol. Chem.*, 1932, **26**, 240.

The results seem to indicate that inositol is a decholesterizing agent and effects some mobilization of cholesterol and cholesterol esters from the tissues.

15628

Prolongation of Penicillin Activity with Penicillinase-Inhibiting Compounds.

ROGER D. REID, LLOYD C. FELTON, AND MIRIAM A. PITTROFF.
From the Research Laboratories, Hynson, Westcott & Dunning, Inc., Baltimore, Md.

Methods for prolonging the activity of penicillin in the body by use of various compounds to delay absorption or excretion have been proposed and reviewed fully in the literature. We have made extensive studies on an oral penicillin preparation composed of alum-precipitated penicillin or aluminum penicillin mixed with sodium benzoate. It has been determined from laboratory and clinical experience that sodium benzoate prolongs the blood serum levels when given with penicillin, either orally or parenterally. Other investigators studying this or similar compounds have explained the prolonged blood serum levels on the basis of renal blockage due to absorption of hippuric acid, formed from such compounds, by the renal tubules. Evidence to support this explanation is unconvincing with the dosage we have used. The literature is not clear as to how much sodium benzoate or benzoic acid is needed to produce sufficient hippuric acid to cause an effective renal blockage. In order to find another explanation we have attempted to demonstrate a mechanism that prevents or delays the destruction of penicillin by enzymes.

Normally the intestine contains a large number of "coliform" organisms that are known to produce penicillinase. If this is true then destruction of penicillin in the intestine might be prevented by (1) inhibition of the penicillinase-producing bacteria, or (2) allowing the bacteria to grow while preventing production of the enzyme, or (3) the destruction or inactivation of penicillinase produced.

The following preliminary experiments were made to clarify these points. To begin with, compounds such as sodium sulfathalidine and bromsaligenin, effective in inhibiting or killing the Gram-negative intestinal bacteria, were used. Substances having no inhibitory effect on the Gram-negative bacilli, such as sodium benzoate, sodium sulfanilate and *p*-amino benzoic acid, were selected for study as representing this group of compounds.

In the *in vitro* experiments flasks containing 100 ml of penicillin assay broth, F.D.A. modification, with 10 units of penicillin sodium (Lilly) per ml were inoculated with 0.2 ml of an 18-hour broth culture of *Escherichia coli.* Drugs were added to individual flasks in concentrations dependent upon their solubilities and estimated strength necessary to produce satisfactory results. Control flasks containing the drugs without penicillin were inoculated, and control flasks of penicillin with and without *Esch. coli* were incubated. Another control series in which the broth contained the penicillin and drug was inoculated with coli and incubated.

All flasks were incubated at 37°C. After

TABLE I.
Protection of Penicillin Against *Esch. coli* Factors *in Vitro.*

		Zone of inhibition of *Staph. aureus* 209-P after 18 hours incubation		
		Drug with 10 u/ml penicillin. Incubated with *Esch. coli* mm	Drug with 10 u/ml penicillin. No bacteria mm	Drug alone mm
Sodium benzoate	1.0%	27	34	0
Bromsaligenin	0.1	34	34	0
Sodium sulfathalidine	1.0	19	33	0
Phenylethyl alcohol	5.0	33	33	—
Sodium lauryl sulfate	0.5	28	32	12
” oleate	0.5	25	33	—
” stearate	0.5	0	32	0
” sulfanilate	1.0	0	32	0
Penicillin control, 10 u/ml			32	
Penicillin, 10 u/ml, incubated with *Esch. coli*			0	

TABLE II.
Protection of Penicillin Against Clarase *in vitro.*

Test	Zone of inhibition of *Staph. aureus* 209-P after 3 hr exposure to Clarase mm	Control	Zone of inhibition of *Staph. aureus* 209-P after 3 hr incubation mm
Sodium benzoate 0.5% Penicillin 10 u/ml Clarase 0.25%	15	Sodium benzoate 1.0% Penicillin 10 u/ml	38
Sodium benzoate 1.0% Penicillin 10 u/ml Clarase 0.25%	18	Sodium benzoate 1.0%	0
Sodium sulfanilate 0.1% Penicillin 10 u/ml Clarase 0.25%	16	Sodium sulfanilate 1.0% Penicillin 10 u/ml	40
Sodium sulfanilate 0.5% Penicillin 10 u/ml Clarase 0.25%	20	Sodium sulfanilate 1.0%	0
Sodium sulfanilate 1.0% Penicillin 10 u/ml Clarase 0.25%	24		
Methionine 0.1% Penicillin 10 u/ml Clarase 0.25%	12	Methionine 0.1 % Penicillin 10 u/ml	38
		Methionine 0.1%	0
Penicillin 10 u/ml Clarase 0.25%	0	Penicillin 10 u/ml	40

18 hours some of the material in each flask was passed through a Swinney filter to remove the bacteria and the filtrate added to the cups on a penicillin assay plate, using the standard F.D.A. technic with *Staphylococcus aureus* 209-P as the test organism. In the inoculated flasks there was no growth of *Esch. coli* if the medium contained bromsaligenin or phenylethyl alcohol, but there was good growth in the other inoculated flasks. Plate assays were made from all flasks regardless of growth of *Esch. coli.*

Table I shows the results of such assays.

The results in Table I indicate that there is some mechanism which interferes with penicillinase activity of *Esch. coli* without necessarily inhibiting the growth of that organism. The results indicate further that the inhibition of *Staph. aureus* 209-P on the test plates is not due to the inhibiting drug alone or to a concerted effect of the drug and penicillin but is due solely to the penicillin.

It appeared from the experiments just described that sodium benzoate may exert an antipenicillinase activity. If this were the case, it might be that factor which accounts for prolonged blood serum levels after administration of penicillin with sodium benzoate. To explore this possibility further, penicillin, penicillin plus sodium benzoate, and penicillin plus other compounds were exposed to the action of preformed penicillinase. If penicillin were protected under this set of conditions, it would indicate that protection is not due entirely to the prevention of penicillinase production. Penicillinase was first obtained by filtering broth cultures of a penicillinase-producing strain of *Esch. coli*. Later Clarase*[1] was used as the source of penicillinase.

Penicillin assay broth (F.D.A. modification)[2] was distributed into flasks in 100 ml amounts. To these 0.25% final concentration of Clarase was added. The various drugs were placed in individual flasks to test the antipenicillinase activity. Controls of the same nature as before were run. Mixtures were incubated at 37°C for 3-4 hours and penicillin assay plates were made as before, using *Staph. aureus* 209-P as the test organism. Results are shown in Table II.

These experiments seemed to indicate that certain compounds had the ability to interfere with the activity of penicillinase (in the form of Clarase) *in vitro*. It was decided to examine a rather large group of chemicals for these effects. The methods used in each instance were as described above. The compounds examined and results are shown in Table III. It will be observed that a number of the tested compounds inhibited *Esch. coli* partially or completely. As a result, there was no destruction of penicillin. These same compounds, however, failed to protect the penicillin from the Clarase activity. On the other hand, there were a few compounds which allowed the bacterium to grow, yet protected the penicillin from the enzyme. These compounds were found to confer protection on the penicillin when exposed to penicillinase enzyme. Selection of certain of these compounds for *in vivo* trial was made on the relative efficiency of the drug in protecting the penicillin against the enzyme *in vitro* and, secondly, upon the lack of toxicity of the drug. On this basis, sodium benzoate and sodium sulfanilate were selected as compounds not affecting growth of the bacteria yet protecting against one or the other enzyme, and bromsaligenin was used because of its bacteriostatic or bactericidal activity against Gram-negative bacilli.

Mice weighing 20 g were injected intraperitoneally with 0.5 ml of a 10^{-5} dilution of an 18-hour broth culture of *Diplococcus pneumoniae* Type I. Two hours later treatment of the mice was begun by intraperitoneal injection of penicillin with or without one of the penicillinase-inhibiting drugs. Treatment consisted of one daily dose of 50 units for each mouse. Final readings were made on the 7th day. Results are shown in Table IV.

There was no evidence of toxicity to sodium sulfanilate in the amounts used which was nearly 5.0 g daily per kg of body weight, This is in line with earlier studies on the toxicity of this compound.[3] There were no toxic effects noted in the case of the mice receiving the bromsaligenin (0.25 g per kg of body weight), although the mice were under the effect of general anesthesia for nearly 30 minutes following each treatment. Sodium benzoate given in the same amount as sodium sulfanilate caused death in the

---

* Clarase—the commercial name for a preparation made by Takamine Laboratories, Inc., which contains an enzyme active against penicillin.

[1] Lawrence, C. A., *Science*, 1943, **98**, 413.

[2] Schmidt, William H., and Moyer, Andrew J., *J. Bact.*, 1944, **47**, 199.

[3] Hebb, Arthur, Sullivan, S. G., and Felton, Lloyd, D., *Public Health Rep.*, 1939, **54**, 1730.

TABLE III.
Effect of Chemical Compounds on Clarase Activity and Activity of Penicillinase Produced by *Escherichia coli in vitro.*

Compound	% conc.	Growth of *Esch. coli*	Zone of inhibition of *Staph. aureus* after exposure to:	
			Esch. coli mm	Clarase mm
Sodium caproate	1.0	+	28	0
’’ stearate	0.5	++	0	0
’’ oleate	0.5	?	25	14
’’ succinate	0.5	+	24	0
’’ adipate	0.1	++++	0	0
’’ lactate	1.0	++++	0	0
’’ tartrate	0.5	?	13	0
’’ citrate	0.5	+	23	0
Calcium gluconate	1.0	+++	0	0
Sodium thioglycollate	0.5	++++	0	0
’’ lauryl sulfate[*]	0.5	++	28	32
’’ stearyl sulfate	0.1	++++	0	0
’’ formaldehyde sulfoxylate	0.5	++++	0	0
’’ glutamate	0.5	+++	0	0
Methionine	0.1	+++	0	0
Barbituric acid	0.1	++++	0	0
Hexamethylene tetramine	1.0	0	20	0
Phenylazo-diaminopyridine mono-hydrochloride	0.1	+++	0	0
Ammonium sulfamate	0.5	?	0	12
Bromsaligenin	0.1	0	34	0
Phenylethyl alcohol	5.0	0	35	—
Sodium mandelate	1.0	+	0	12
’’ benzoate	1.0	++++	27	18
’’ *o*-chlorbenzoate	0.5	++++	0	0
’’ salicylate	1.0	0	30	0
’’ *p*-hydroxybenzoate	0.5	++++	0	0
’’ dibromsalicylaldehyde[†]	0.5	?	29	12
’’ anthranilate	0.5	+++	0	0
’’ *p*-aminobenzoate	1.0	++++	0	0
’’ *o*-sulfobenzoate	1.0	++++	11	0
’’ benzene sulfonate	1.0	++++	0	16
’’ *p*-chlorphenol sulfonate	0.5	0	28	20
’’ sulfanilate	1.0	++++	0	24
Sulfanilic acid	0.2	++++	0	16
Sodium acetyl sulfanilate	1.0	++++	0	20
Acetyl sulfanilic acid	1.0	++	0	20
Sodium dibrom sulfanilate	1.0	+++	13	17
’’ sulfanilamide	0.1	++++	0	0
’’ sulfathiazole	0.1	++++	0	0
’’ sulfathalidine	1.0	+++	19	—
p-Carboxybenzene sulfonamide	0.5	+++	0	0
Sodium *β*-resorcylate	1.0	++++	13	0
’’ di-iodo-*β*-resorcylate	1.0	?	26	12
’’ cholate	0.1	++++	0	0

[*] Sodium lauryl sulfate alone produced a zone of 12 mm.

[†] Sodium dibromsalicylaldehyde alone produced a zone of 11 mm.

mice within 30 minutes. Reducing the amount of sodium benzoate to .009 g per dose was not toxic for mice but failed to give added protection. It will be observed that a combination of penicillin and sodium sulfanilate, neither of which alone afforded adequate protection against pneumococci, gave complete protection to these mice. A combination approaching this in effectiveness was penicillin and bromsaligenin. So far as could be determined from the *in vitro* experiments, this effect of the latter drug was solely due to the inhibition of *Esch. coli.*

Rabbits were given penicillin alone and in combination with sodium sulfanilate, sodium benzoate or bromsaligenin. The rabbits

TABLE IV.
Effect of Penicillinase-inhibitors *in vivo.*

Treatment	Total dose	Deaths	Survivors
Penicillin, 50 u/day	200 units		
Sodium sulfanilate, .095 g/day	.37 g	0	20
Penicillin, 50 u/day	200 units		
Sodium benzoate, .0095 g/day*	.04 g	9	1
Penicillin, 50 u/day	200 units		
Bromsaligenin, .0045 g/day	.022 g	4	16
No treatment (controls)	0	20	0
Penicillin, 50 u/day	200 units	16	4
Sodium sulfanilate, .095 g/day	.19 g†	10	0

* There were 20 mice in this group to begin with, all of which died within an hour of the first dose of penicillin (50 units) containing .095 g of sodium benzoate. A second group of only 10 mice was then treated with 0.1 the amount of sodium benzoate, or .0095 g daily, as indicated in the table.

† These mice died before receiving a third dose of the drug.

weighed approximately 1.5 kg and the dose of penicillin in each case was 2000 units. Animals were bled at intervals of 1 to 1½ hours and blood serum levels were determined by the technic of Randall, Price and Welch[4] with modifications suggested by Chandler, Price and Randall.[5]

Sodium sulfanilate given with penicillin, intravenously, gave rise to somewhat prolonged blood serum levels of penicillin, whether the sodium sulfanilate was given intravenously or by mouth. Penicillin given intravenously with bromsaligenin by mouth also gave slightly prolonged penicillin levels in blood.

Discussion. In prolonging the activity of penicillin in the body, one or more of a number of factors may play an important part. *Delayed absorption* may be accomplished by injections of mixtures of penicillin with oil,[6] peanut oil and beeswax,[7] gelatin,[8] or alum[14] or by oral administration with such compounds as aluminum hydroxide gel.[9] Delayed absorption has also been attempted by the use of ice packs at the site of injection.[10]

Methods for *delaying excretion* that have been proposed include the use of penicillin with para-aminohippuric acid,[11] diodrast,[12] benzoic acid[13] and sodium benzoate.[14,15]

Acid-neutralizing agents for combination with penicillin for oral use have been incorporated into many preparations, usually in the form of buffers.

McDermott *et al.*[16] have pointed out that the advantage in protecting penicillin against stomach acidity, "is limited to the difference between the amount of absorption which occurs in the absence of such protection and the maximal absorption when acid destruction is not a factor." Since this amount is very small, they reason that no significant superiority will be noted when an acid-resistant salt of—or acid-protected penicillin is used, over one not so protected.

It therefore appears that any superiority of an oral penicillin preparation, and indeed a penicillin preparation for parenteral use, will be dependent upon some characteristic of the penicillin itself, or upon its combination with some substance which will protect it against agents in the intestinal tract or body fluids and tissues that destroy or inactivate it.

---

[4] Randall, William A., Price, Clifford W., and Welch, Henry, *Science,* 1945, **101**, 365.

[5] Chandler, Velma L., Price, Clifford W., and Randall, William A., *Science,* 1945, **102**, 355.

[6] Raiziss, G. W., *Science,* 1944, **100**, 412.

[7] Romansky, M. J., and Rittman, G. E., *Science,* 1944, **100**, 196.

[8] Parkins, W. M., Wiley, M., Chandy, J., and Zintel, H. A., *Science,* 1945, **101**, 203.

[9] Welch, Henry, Price, Clifford W., and Chandler, Velma L., *J. A. M. A.*, 1945, **128**, 845.

[10] Trumper, M., and Hutter, A. M., *Science,* 1944, **100**, 432.

[11] Beyer, Karl H., Woodward, Roland, Peters, Lawrence, Verway, W. F., and Mattis, P. A., *Science,* 1944, **100**, 107.

[12] Rammelkamp, C. H., and Bradley, S. E., PROC. SOC. EXP. BIOL. AND MED., 1943, **53**, 30.

[13] Bronfenbrenner, Jack, and Favour, Cutting B., *Science,* 1945, **101**, 673.

[14] Bohls, S. W., and Cook, E. B. M., *Texas State J. Med.*, 1945, **41**, 342.

[15] Bohls, S. W., Cook, E. B. M., and Potter, R. T., *J. Ven. Dis. Inform.*, 1946, **27**, 69.

[16] McDermott, Walsh, Bunn, Paul A., Benoit, Maria, DuBois, Rebeckah, and Reynolds, Mildred E., *Science,* 1946, **103**, 359.

In the case of oral penicillin preparations, the amount that may be absorbed under the most favorable conditions is dependent upon the protection of penicillin, while in the intestine, from destruction by bacteria or their enzymes. We have demonstrated in the foregoing experiments that protection against the most common of these agents, penicillinase, can be had by a combination with sodium sulfanilate, sodium benzoate or certain other compounds. The mechanism of this protection cannot be explained at this time, since related compounds (chemically) fail to confer this protection.

It has further been observed that certain compounds, such as bromsaligenin, that do not interfere with enzymatic activity may produce a similar result by inhibiting the penicillinase-producing bacteria.

It can be shown that sodium sulfanilate or sodium benzoate protect penicillin to some extent against penicillinase *in vitro*. If breakdown of the penicillin after absorption is accomplished by enzymes in body tissues and fluids (and this seems likely since there is a considerable loss of penicillin even after parenteral administration), it seems possible that this, too, may be reduced by the protective effect of these drugs.

Conclusions. 1. Penicillin blood serum levels can be prolonged by the administration of penicillin with sodium sulfanilate, sodium benzoate, bromsaligenin or certain other compounds. 2. Effect of bromsaligenin on penicillin has been demonstrated *in vitro* to be due to inhibition of growth of *Esch. coli* and other Gram-negative intestinal bacteria. 3. The effect of sodium benzoate or sodium sulfanilate on penicillin has been shown *in vitro* to be due to inhibition, at least temporarily, of the enzyme penicillinase. 4. That such drugs are useful and effective in prolonging penicillin levels in the blood and so increasing the efficiency of penicillin has been shown by *in vivo* tests in mice and rabbits. 5. A list of compounds that do not possess this quality has been given, as well as some chemicals that give questionable results.

15629

Action of Penicillin on Staphylococcus. Effect of Size of Inoculum on the Test for Sensitivity.*

R. F. PARKER.

From the E. H. Cushing Laboratories of Experimental Medicine, Lakeside Hospital, and the Department of Medicine, Western Reserve University Medical School, Cleveland, Ohio.

In some of the early reports[1,2] on the antibacterial effect of penicillin, it is stated that the action of this agent appears to be unrelated to the number of bacteria present. Perhaps as a result of this, the commonly used tests[3,4] for penicillin susceptibility of organisms vary widely from laboratory to laboratory in respect to the size of the inoculum which is used. In the course of certain other work

---

* This work was supported in part by The Upjohn Company.

[1] Abraham, E. P., Chain, E., Fletcher, C. M., Gardner, A. D., Heatley, N. G., Jennings, M. A., and Florey, H. W., *Lancet*, 1941, **241**, 177.

[2] Hobby, G. L., and Dawson, M. H., PROC. SOC. EXP. BIOL. AND MED., 1944, **56**, 178.

[3] Rammelkamp, C. H., and Maxon, T., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 386.

[4] Spink, W. W., and Ferris, V., *Science*, 1945, **102**, 221.

TABLE I.
Penicillin Inhibition of Staphylococcus.

Penicillin per ml	"Susceptibility Ratio" (see text)													Total
	0	1	2	3	4	5	6	7	8	9	10	11	12	
64.		1	1											2
32.														
16.														
8.														
4.		3												3
2.														
1.														
.50							1		10	5				16
.25			1	1		1		1	2	2	1	2		11
.12	3	2	1	1					1				2	10
.06	5	40	14		3			1						63
.03	13	13	12	1										39
.016		8	6	1	2									17
.008			1	1										2
Total	21	67	36	5	5	1	1	2	13	7	1	2	2	163

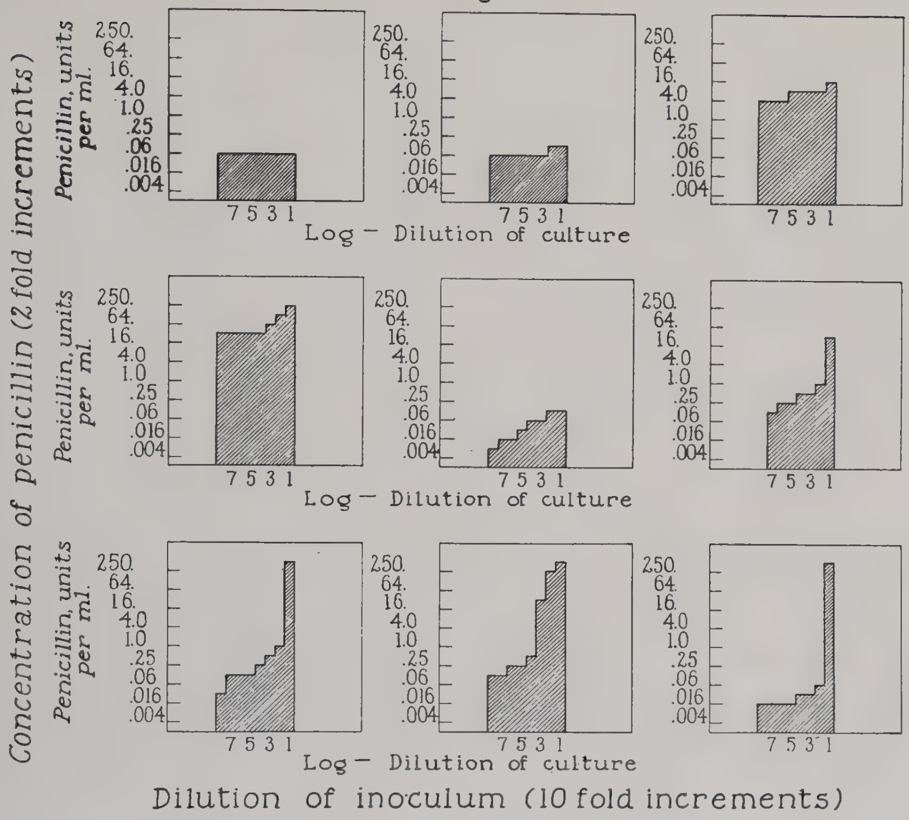
in this laboratory on the action of penicillin on Staphylococcus it was observed that the antibacterial action of penicillin was not always independent of the density of the bacterial population, but that the results of penicillin sensitivity tests might be profoundly influenced by the size of the inoculum. Because of the great practical as well as theoretical importance of the question it was decided to explore the problem systematically.

One hundred and sixty-three strains of *Staphylococcus aureus*, isolated in the routine diagnostic laboratory serving Lakeside Hospital, were subjected to tests of penicillin susceptibility as follows. A series of culture tubes was prepared, having in each 0.5 ml of tryptose phosphate broth, and containing penicillin in concentrations from 1000 to 0.002 units per ml. Two such series were used for each organism tested, one series being inoculated with 0.5 ml of a 10^{-2} dilution of a 48-hour culture in tryptose broth and one with a 10^{-6} dilution. They were incubated overnight, and the tube in each series containing the minimal inhibitory concentration of penicillin was identified. The viable cell count of the 48-hour broth cultures used for inoculation was Log 8.85 (S.D. 0.65) per ml, the inoculum therefore Log 2.85 and Log 6.85 on the average. The concentration of penicillin giving complete inhibition of growth in the small-inoculum series was arbitrarily taken as the "susceptibility" of the organism. The discrepancy, if any, between the 2 tests was recorded as the number of tubes (*i.e.* 2-fold increments of penicillin concentration) by which the end-points differed. Thus an organism, growth of which was prevented by 0.06 units/ml when using a small and 0.25 units/ml when using the large inoculum was recorded as having a penicillin susceptibility of 0.06-2. The results of these tests are collected in Table I.

It is seen from the totals given in the last column of Table I that the distribution of the organisms with regard to penicillin susceptibility approaches normality, although there is a definite suggestion that 2 populations are involved. Of these, the one might have a peak at susceptibility to 0.06 unit per ml of penicillin, the other at susceptibility to 0.50 unit per ml.

In the bottom row of Table I will be found the distribution of organisms according to the "susceptibility ratio." This is the ratio between the results of the 2 tests of susceptibility, expressed as the number of tubes in the series which separate the end-points of the determinations. Here there are evidently 2 distinct populations. In one, the outcome of the test of penicillin sensitivity is influenced little if at all by the number of organisms inoculated; in the other, the influence is striking. The separateness of the 2 groups of organisms is revealed also in

Fig. 1.



the correlation table itself, for there are evidently 2 centers around which the data tend to cluster. In one of these the organisms have low resistance, and a low ratio between the results of high- and low-inoculum tests; in the other definitely greater resistance to penicillin is associated with a high ratio.

All of the strains studied were subjected to the duplicate test of penicillin sensitivity. In addition, a certain number were subjected to a more elaborate one. In this test, replicate determinations of penicillin sensitivity were performed, using as inoculum serial 10-fold dilutions of culture from 10^{-1} to 10^{-8}. For each inoculum the minimal inhibitory concentration of penicillin was recorded. A representative group of the results are collected in Fig. 1, inspection of which reveals that it is possible to arrange the histographs in a fairly regular sequence.

At one extreme are found those strains in which the result has no relation to the size of the inoculum. At the other extreme are those in which stepwise increase in size of inoculum results in a stepwise increase in the apparent resistance of the organism to penicillin, with progress from one extreme to the other being made gradually.

While this study was in progress, Luria[5] called attention to the fact that the results of the dilution test of sensitivity to penicillin might be profoundly influenced by the number of organisms used in the inoculum. The technic which he proposed for testing penicillin sensitivity is essentially the one used here, and these results may therefore be interpreted as an extension of his, and confirmation of his prediction that the phenomenon was not rare. In Luria's opinion those organisms which showed a marked discrepancy in the results of the 2 tests were those which were able to elaborate penicillinase. The data reported here give no direct information on this point, but the variety of curves obtained when resistance to penicillin is plotted against regular increases in the number of cells in the inoculum suggests that the phenomenon may have a complex basis. That this rule may not always hold is suggested by the fact that one organism, resistant to penicillin and capable of producing penicillinase showed only a slight difference in apparent sensitivity in the 2 tests. Further work on this line is now in progress.

---

[5] Luria, S. E., Proc. Soc. Exp. Biol. and Med., 1946, **61**, 46.

On the other hand, that in regard to this character there are 2 sorts of staphylococci seems apparent. It is of considerable importance clinically that the great bulk of the organisms fall in the group against which the antibacterial action of penicillin is exerted with little regard to the actual density of the bacterial population.

Conclusions. 1. The action of penicillin *in vitro* against most strains of Staphylococcus is related only in a minor degree to the number of bacteria present. 2. In a not inconsiderable group of strains, somewhat more resistant to penicillin than the majority, the outcome of tests for penicillin sensitivity is profoundly influenced by the number of cells present.

15630

Clinical Trial of Gamma Globulin in the Prevention of Common Respiratory Diseases.

JOHN M. ADAMS AND NATHAN SMITH.

From the Department of Pediatrics, University of Minnesota, Minneapolis.

Serum gamma globulin, separated and concentrated by chemical fractionation of normal human blood, was administered at monthly intervals to one-half of a group of 70 students throughout the respiratory disease season from October, 1945, to May, 1946. The incidence of acute respiratory disease, including whatever the individual considered as a "cold" of any degree of severity was carefully tabulated in the entire group.

The chemical fractionation of human blood plasma has made available certain fractions of globulin which appear to contain many specific neutralizing antibodies. Its value in the prophylaxis of measles[1,2] is now common knowledge in spite of the fact that it is still impossible to measure the concentration of measles protective substance directly. The potency of Fraction II (largely gamma globulin) was shown by Enders[3] in the case of neutralizing antibody for influenza A virus to approximate that of sera taken during early convalescence from the disease. Its protective value in clinical trial studies has not been demonstrated to our knowledge. The subjects in our study passed through an influenza epidemic but the numbers in both groups contracting the disease in a clinically recognizable form were so small in each that no conclusions can be drawn as to its prophylactic value in this instance. Enders[3] found Fraction II to contain antibodies reacting with diphtheria toxin, streptococcal erythrogenic toxin, influenza A virus, mumps virus, and the H antigen of *E. typhosa*. These antibodies he found to be concentrated from 15 to 30 times in the fraction as compared to pooled plasma. From pools of plasma obtained from several areas throughout the United States, a satisfactory degree of uniformity between the various preparations was demonstrated.[3] The gamma globulin used

---

[1] Ordman, C. W., Jennings, C. G., and Janeway, C. A., *J. Clin. Invest.*, 1944, **23**, 541.

[2] Stokes, J., Jr., Maris, E. P., and Gellis, S. S., *J. Clin. Invest.*, 1944, **23**, 531.

[3] Enders, John F., *J. Clin. Invest.*, 1944, **23**, 510.

in the present study was furnished on request by the Cutter Laboratories.

Clinical trials of gamma globulin are probably the most practical method of evaluating its direct prophylactic effect. This study was undertaken primarily to determine whether or not the concentrated gamma globulin contained some protective substances against common respiratory diseases. Our previous studies[4,5] indicated that prematurely born infants were much more susceptible than full-term infants to an epidemic respiratory disease presumably caused by a pneumotropic virus. There was also some clinical evidence that pooled adult serum and gamma globulin were effective in prevention or amelioration of this disease. Older children and adults did not appear to be susceptible to it. If gamma globulin could be shown to be even partially effective in preventing the occurrence of common respiratory diseases, such knowledge would indicate that man is capable of building neutralizing substances to some of these diseases. The difficulties encountered in any attempt to evaluate the many varied and undifferentiated respiratory diseases were appreciated from the outset and consequently this material is presented with tentative conclusions only.

Methods. A single class of medical students was selected because it was felt that close cooperation of the type required for the study could best be attained from such subjects. The students were living together every day in classes and laboratories in intimate association. A careful history as to their past experience with acute respiratory disease was obtained, and from these data the entire group was divided into 3 classes as to frequency of disease. One-half of each of these 3 classes was selected as experimental subjects. In October an initial dose of 6 cc of concentrated gamma globulin solution was administered intramuscularly to each student in the experimental group. Thereafter, 4 cc of the same solution was given at monthly intervals, most of the subjects receiving a total of 30 cc in 7 inoculations.

The occurrence of attacks of respiratory disease, their severity and the incidence of reactions to the inoculations as manifested by sore arm, fever or other abnormal manifestations were determined by frequent interviews with each student.

Results. In the experimental group of 35 subjects, 77 episodes of acute respiratory disease were recorded for the season. The control subjects experienced 132 illnesses during the same period. The average number of attacks per person was 2.2 in the experimental group as compared with 3.8 in the control subjects. The previous season experiences of the 2 groups are compared in Table I.

TABLE I.
Data on the Incidence of Acute Respiratory Diseases in the Experimental and Control Subjects.

	Test season		Previous season	
	Exp.	Control	Exp.	Control
Total infections	77	132	125	128
Avg per person	2.2	3.8	3.57	3.65

In the experimental group, the diseases were classed as severe in 10 instances, moderate in 17, the remainder being considered mild. Their previous season record showed that 28 attacks were considered to be severe, 38 moderate, and the remainder mild, Accordingly there was a substantial reduction in severity as compared with the previous season for this group (Table II). Not all subjects were able to classify their illnesses accurately.

TABLE II.
Data on the Severity of Infection in the Experimental Group.

Infections recorded as	Exper. group	
	Test season	Previous season
Severe	10	28
Moderate	17	38
Mild	49	46

During the 1945 influenza epidemic, 7 of the experimental subjects had influenza. This was mild in all instances. Among the control subjects there were 11 cases of influenza during the same period. One subject was hospitalized because of severity of his illness. About one-third of the students

---

[4] Adams, J. M., Green, R. G., Evans, C. A., and Beach, N., *J. Ped.*, 1942, **20**, 405.

[5] Adams, J. M., *Journal-Lancet*, 1945, **65**, 192.

in this study were vaccinated against influenza A and B. An analysis of those who had not received gamma globulin shows a slight reduction in the incidence of respiratory disease, but the group was too small to warrant conclusions.

Reactions consisted primarily of a sore arm at the site of the injection, lasting for one day only in most of the 26 subjects who reported a reaction. Nine reported a sore area about the site for 2 days, and 8 for 3 days. These reactions were extremely variable and recorded for the most part with the first injection only. A low grade fever was recorded in but 3 patients in the entire series and this occurred after one injection only. The highest temperature was 100°F. There were no instances of jaundice in the series receiving the gamma globulin.

Discussion. Too little is known of man's ability to produce neutralizing antibodies active against the common respiratory diseases, many of which are undoubtedly due to viruses as yet unclassified. Gamma globulin has been shown to contain many neutralizing antibodies and to have protective and prophylactic effect in certain specific diseases such as rubeola[1,2] and infectious hepatitis.[6] Our interest in the possibility that immunity might be developed against primary respiratory disease had its origin in the observation that prematurely born babies were much more highly susceptible to a specific respiratory disease than were normal full-term infants.[4] In addition we have never observed the specific disease referred to in older children or adults, at least in the form of pneumonitis. Unpublished evidence indicates that a mother suffering from a common respiratory infection at the time of delivery, may infect her offspring and the baby may show definite clinical and X-ray signs of pneumonitis shortly after birth. This fact is well established in the case of measles (rubeola). It has been shown that passive transfer of antibodies to the fetus probably takes place largely during the last few weeks or months of pregnancy. The immature fetus is much more susceptible to certain viruses than the full-term baby, as has been successfully demonstrated in animals in the case of influenza virus.[7,8]

A study such as that reported here needs to be repeated with a much larger group. Nevertheless it seems possible at present that preliminary clinical trials such as this may throw some light on a question concerning which we need more information, namely, the degree to which man is able to form neutralizing antibodies to some of the common respiratory diseases. Yannet and Deutsch[9] report failure in a group of 25 patients who developed an unknown epidemic respiratory disease (influenza-like) after having received but one prophylactic injection of gamma globulin. No mention is made in their report as to the severity of the disease in the patients receiving gamma globulin as compared with those who did not receive it. They state, "That there proved to be no evidence of prophylactic value of gamma globulin for this type of respiratory infection is not surprising." On the contrary, it seems surprising that there was not some evidence of prevention from concentrated gamma globulin obtained from a large pool of donors, if the disease they were dealing with was a common and not a rare respiratory disease. The possible advantage of repeated monthly injections as employed in the present study is an interesting point about which to speculate. The accumulative effect of passive transfer of antibodies may be important in a disease to which man may form a partial degree of temporary immunity. In our study the majority of the infections were recorded in the first half of the season as opposed to the last half. This fact may be due entirely to the epidemic character of respiratory diseases. We hope to be able to follow this approach to the problem of immunity in this obscure and difficult group of diseases further.

Summary. Concentrated gamma globulin

---

[6] Stokes, J., Jr., and Neefe, J. R., *J. A. M. A.*, 1945, **127**, 144.

[7] Woolpert, O. C., Gallagher, F. W., Rubinstein, L., and Hudson, N. P., *J. Exp. Med.*, 1938, **68**, 313.

[8] Dettwiler, H. A., Hudson, N. P., and Woolpert, O. C., *J. Exp. Med.*, 1940, **72**, 623.

[9] Yannett, H., and Deutsch, J. V., *J. A. M. A.*, 1946, **131**, 593.

was administered at monthly intervals to one-half of a group of 70 medical students throughout a single respiratory disease season from early October to late May. The incidence of attacks of acute respiratory disease was reduced 40% in the experimental subjects as compared with the controls. The severity of the illnesses was significantly decreased in the experimental subjects as compared with their previous season experience.

The reactions to the injections were mild and consisted primarily of local soreness at the site, lasting one day in the majority of the students. No jaundice or hepatitis was observed.

The primary object of this small clinical trial of concentrated gamma globulin was not to discover a method of preventing colds, but to determine if possible, whether or not man is capable of forming neutralizing substances for some of the undifferentiated respiratory diseases. A cumulative effect of repeated injections was suggested by the results.

A statistical evaluation of the data shows a 2% or less probability of error, which would indicate that the results were significant.

15631

Effect of Types of Treatment on Development of Antistreptolysin in Patients with Scarlet Fever.*

Louis Weinstein and Clifford C. L. Tsao.† (Introduced by Chester S. Keefer.)

From the Haynes and Evans Memorials, Massachusetts Memorial Hospitals, and the Department of Medicine, Boston University School of Medicine, Boston, Mass.

During the course of studies on the treatment of scarlet fever with various agents, a somewhat greater incidence of recurrent pharyngitis, with or without a scarlatiniform eruption, was noted in patients treated with penicillin as contrasted with those receiving no therapy or globulin. For this reason, a study of the antistreptolysin titer in the sera of patients with this disease treated by the 3 different methods was carried out. Although it is well recognized that there is no relation between the level of antistreptolysin in the serum and the total antistreptococcal immunity of an individual, studies of this nature are of value, however, in reflecting the response of a host to experience with the beta hemolytic streptococcus. In a similar study, Rantz, Boisvert, and Spink[1] found that the administration of salicylates, sulfadiazine, or a "short course" of penicillin had no effect on the mean increase in antistreptolysin. When penicillin was administered over a longer interval, the frequency of antifibrinolysin response was decreased and an antistreptolysin response was also observed less often in the patients in whom bacteriologic relapse did not occur.

Methods. The cases were chosen for each type of treatment alternately and without respect to the severity of their disease. One group received only symptomatic treatment, another human gamma globulin intramuscularly (1 cc per pound of body weight up to 60 cc), and a third was treated with 120,000 units of penicillin daily for 10 days. Cultures of the nose and throat on blood-heart infusion agar and in blood broth were made daily for the first week and every other day thereafter. Serums for determination of antistreptolysin titers were drawn on the day of admission and every 7 days thereafter as long as the patient was in the hospital; a minimum of 3 serums were studied in each case, the last one being obtained at least 21 days after the start of the infection. Antistreptolysin titers were determined by the method of Rantz and Randall.[2] Only a dif-

---

* The gamma globulin used in this study was supplied by the American Red Cross.

† Visiting Fellow to the Haynes Memorial Hospital, and the Department of Medicine, Boston University School of Medicine, from the Department of Medicine, West China Union University Medical College, Chengtu, China.

[1] Rantz, L. A., Boisvert, P. J., and Spink, W. W., *Science*, 1946, **103**, 352.

TABLE I.
Effect of Various Types of Treatment on Development of Antistreptolysin in Patients with Scarlet Fever.

Type of treatment	Cases with rise in antistreptolysin titer		Avg antistreptolysin titer incr., Units	Cases without rise in antistreptolysin titer	
	No.	%		No.	%
Symptomatic	18	72	300	7	28
Conc. human globulin	9	64	279	5	36
Penicillin	4	13	189	29	87

ference of at least 2 tubes in titration of antistreptolysin was considered as significant.

Results. Twenty-five patients who were given only symptomatic treatment were studied. Of these 18 (72%) developed a rise in antistreptolysin titer, the average increase being 300 units. Seven patients (28%) had no change in antistreptolysin. Of 14 patients treated with globulin, 64% showed an increase in antistreptolysin, with an average of 279 units. The situation was quite different in 33 patients who received 1,200,000 units of penicillin over a period of 10 days; only 4 (13%) showed an antibody response, the average rise being considerably lower than that in the other 2 groups, 189 units. Eighty-seven per cent of the penicillin-treated patients exhibited no increase in antistreptolysin.

Analysis of the patients with respect to development of complications revealed that of the 18 untreated ones who had shown a rise in antistreptolysin titer, 2 developed glomerular nephritis, 2 had recurrent fever without evidence of a localized process, and 1 had proved rheumatic fever. Of the 7 untreated cases who showed no increase in antistreptolysin, 2 had recurrent streptococcal pharyngitis and 1 recurrent scarlet fever. In the globulin-treated group, 2 of the 9 patients who showed an increased antistreptolysin content were classified as rheumatic fever suspects, 1 developed proved rheumatic fever, and 6 showed no complications. None of the globulin-treated individuals who failed to show an antibody response suffered any complications other than cervical lymphadenopathy with fever. Three of the 9 penicillin-treated cases who developed an increased antistreptolysin titer showed recurrences of Group A *Streptococcus pyogenes* in the pharynx without any other manifestations, and 1 was classified as a rheumatic fever suspect. Of the 29 penicillin-treated cases with no rise in antistreptolysin titer, 3 had a recurrence of *Strept. pyogenes* in the pharynx; in 2 of these, fever was present at the same time.

It is interesting to note that all the cases of rheumatic fever, proved or suspected, and of glomerular nephritis occurred in patients who developed a rise in antistreptolysin titer regardless of the type of treatment received. There appeared to be no relation between the recurrence of streptococci in the pharynx and the antistreptolysin response. In the penicillin-treated group, the incidence of recurrence of streptococci in the pharynx was as great in those who showed an increase in antistreptolysin as in those who did not.

Discussion. These data (Table I) suggest that treatment of patients with penicillin for a fairly long period of time results in a decrease in their ability to form antistreptolysin. Although it is possible that this may reflect a generally decreased formation of antibodies, no conclusions can be drawn concerning the effect of various types of treatment on the total immunologic response to streptococcal infection. As the groups studied were very small, more information obtained from a larger number of patients is necessary.

Conclusions. The administration of concentrated human serum globulin to patients with scarlet fever appears to have no effect on their ability to form antistreptolysin. Treatment with penicillin results in a decrease in the number of persons capable of developing this antibody, and also in the quantity of antibody produced by those who show an increase in antistreptolysin titer.

---

[2] Rantz, L. A., and Randall, E., Proc. Soc. Exp. Biol. and Med., 1945, **59**, 22.

15632

Toxicity and Influence of Streptomycin in Breeding of Mice.

CHARLES A. SLANETZ. (Introduced by H. D. Kesten.)

From the Department of Animal Care, Columbia University, New York.

Since preliminary observations[1] in this laboratory indicate that streptomycin* may be a practical means of controlling Salmonella infections in colonies of mice, it was thought desirable to determine what influence streptomycin may have on breeding of mice, weight gains of young mice, and on the general condition of the animals. In addition to observing any obvious toxic effects of this antibiotic when given orally over considerable periods of time, studies of such indirect effects as changes in the intestinal flora were also planned.

Experimental. Young adult Swiss albino mice were obtained for the breeding phase of the work from Rockland Farms.† Fecal samples from each mouse were tested for presence of Salmonella organisms and any giving a positive test were eliminated. Females in good condition which had produced at least one litter of mice were selected and mated in metal cages, one male to 2 females. When the mice were obviously pregnant, they were placed in separate cages. Litters and parent stock were weighed at weekly intervals over a period of 4 weeks. The young males and females of the litters, were weighed individually at 3 and 4 weeks of age. The young were weaned when 3 weeks old. During the entire experiment the basal ration was Rockland Mouse Diet. Streptomycin was incorporated in the diet so that the daily intake of each mouse of 5 groups consisting of 20 females and 10 males each was approximately 1000, 400, 200, 100, and 50 μg respectively. Three additional groups were given 200, 100, and 50 μg per mouse daily in drinking water. The young mice were kept on the same diet as their parents after weaning, until the end of the weighing

TABLE I.
Influence of Streptomycin on Breeding, Number of Mice Per Litter at Birth and at Weaning.

			Avg No. of mice per litter	
μg basal diet	No. of litters born	Sterile females	At birth	Weaned
	First Litters.			
1000	14	6	7	6.0
400	16	4	7	6.0
200	15	5	8	8.0
100	18	2	8	7.6
50	17	3	6	5.9
Controls	15	5	6	5.6
	Second Litters.			
1000	4	1	6	6.0
400	4	0	9	9.0
200	10	3	8	7.0
100	10	3	5	4.5
50	10	3	9	8.0
Controls	14	2	7	7.0
	First Litters.			
200 units H_2O	17	3	9	7.9
100 ,, ,,	16	4	7	6.2
50 ,, ,,	19	1	7	5.8

---

[1] Slanetz, C. A., PROC. SOC. EXP. BIOL. AND MED., 1946, **62**, 248.

* We are greatly indebted to Chas. Pfizer & Co., Brooklyn, N.Y., for the streptomycin used in these studies, and to Dr. Chester S. Keefer, Committee on Chemotherapeutics and Other Agents, National Research Council.

† This work was conducted under the Rockland Farms Research Fund.

TABLE II.
Influence of Streptomycin on Weight Increase in Mice.

	Litter No.	7	14	21 ♀	21 ♂	28 ♀	28 ♂	Age in days
		First Litters.						
1000 μg daily basal ration	1	3.1	6.1	8.5	9.5	14.0	15.1	
	1A	2.3	4.0	8.0	7.0	9.5	11.0	
	2A	1.8	4.5	7.5	7.5	12.0	12.5	
	3	3.1	4.8	9.5	6.5	11.8	11.9	
	3A	3.0	4.1	4.8	5.0	9.4	11.0	
	4A	2.3	5.3	7.5	8.5	12.0	12.5	
	5	3.3	4.2	7.3	7.1	11.3	13.8	
	5A	2.3	4.6	4.5	5.9	9.5	12.3	
	8A	2.8	5.5	9.0	8.5	11.8	14.5	
	9	3.7	5.9	7.5	7.0	8.5	8.5	
	9A	4.5	5.3	9.8	10.5	17.5	13.5	
	10A	2.1	3.6	5.5	4.0	10.3	9.0	
	Avg	2.9	4.8	7.5	7.3	11.4	12.1	
		Second Litters.						
Controls	85A	1.9	4.8	7.1	7.1	11.1	12.0	
	86A	2.3	3.7	7.3	5.5	11.5	8.5	
	87	1.3	3.5	4.0	4.3	8.5	9.0	
	88	3.1	4.0	6.1	6.1	11.5	10.7	
	88A	2.5	3.9	6.0	6.0	9.7	11.7	
	89	3.0	4.2	7.0	6.5	11.3	9.0	
	89A	5.2	6.8	10.5	10.5	16.5	15.5	
	90	3.1	5.0	7.8	7.5	13.2	13.5	
	Avg	2.8	4.5	7.1	6.7	11.4	11.2	

TABLE III.
Averages of Weight Increases in First and Second Litters of Mice Fed Various Levels of Streptomycin in Basal Ration.

μg daily	Litter	7	14	21 ♀	21 ♂	28 ♀	28 ♂	Age in days
1000	1st	2.9	4.8	7.5	7.3	11.4	12.1	
1000	2nd	3.7	6.0	8.6	8.6	13.8	14.2	
400	1st	2.5	4.5	6.4	6.8	9.9	10.1	
400	2nd	3.2	4.4	5.6	6.2	10.1	12.3	
200	1st	2.2	4.5	5.7	6.1	8.9	9.6	
200	2nd	2.8	5.3	7.0	7.4	12.0	12.8	
100	1st	2.2	4.6	6.8	7.4	9.5	11.2	
100	2nd	3.0	5.8	8.2	8.6	10.2	13.5	
50	1st	2.3	4.9	6.0	7.7	9.7	10.5	
50	2nd	2.7	4.9	6.5	6.5	9.7	10.9	
Controls	1st	3.2	5.1	6.8	7.3	10.4	12.5	
”	2nd	2.8	4.5	7.1	6.7	11.4	11.2	

period.

Records were kept as to the number of mice in each litter at birth and at weaning. Table I presents the results obtained at the several levels of streptomycin feeding. Table II shows average weight gains of litters in 2 representative groups of mice at 1, 2, 3, and 4 weeks of age. One week after weaning of their first litters, a number of mice were selected from each group and again mated. The litters of mice obtained from these females are referred to as second litters and weight gains on these litters are given in Table III along with average weight gains of the first litters. At weekly intervals the streptomycin-fed mice were carefully examined as to condition of hair, eyes, tails, and feet, and compared with the control mice fed only the stock mouse diet.

Discussion. The general condition of the streptomycin-fed mice was similar to that of the control mice kept on the stock mouse diet.

There was no evidence of toxicity even in the mice fed 1000 μg of streptomycin daily for 4 to 6 months. No significant difference was apparent between the streptomycin-fed mice and controls in regard to percentage fertility; size of litters and number of mice weaned. The first litters of mice fed various levels of streptomycin showed no significant differences in weight gains but the second litters at the higher levels made greater gains as a rule than controls and those at low level intakes.

First Litters. Weight gains of first litters from mice fed streptomycin were similar to those of control mice fed stock diet.

Second Litters. The mice fed at a daily level of 1000 μg of streptomycin made greater gains than control mice and those given 50 μg daily. At the 400, 200, and 100 μg levels, the trend toward greater weight gains is evident (Table III). A possible explanation for the greater gain in weight of second litters may be that the mothers had obviously been on the streptomycin-containing diet longer, at the time of their birth than when the first litters were produced. Work is now in progress to check the influence of the 1000 μg level as well as 3000, and 6000 μg daily intake of streptomycin on weight gains of young litters of mice.

No gross pathology was evident in several mice sacrificed and autopsied from each group. Histological examination‡ of tissues from mice fed 1000 μg of streptomycin daily revealed no significant changes.

A study of the intestinal bacterial flora of mice receiving 1000 μg of streptomycin daily and of control mice is in progress.

Summary. No toxic effects were observed in albino mice fed streptomycin at daily levels of 1000, 400, 200, 100, and 50 μg for periods of one to 6 months. These levels had no significant influence on percentage fertility, litter size, number of young mice weaned, and general condition of the animals. Although no influence was evident at the various levels of streptomycin in the weight gains of first litters of mice, a greater increase in weight occurred in second litters at higher levels compared to second litters of the control and 50 μg groups.

---

‡ The author wishes to thank Drs. A. M. Pappenheimer and H. C. Stoerck for examination of histological sections.

15633

Histamine and Anaphylactic Shock in the Mouse.*

Seymour M. Perry and Marvin L. Darsie, Jr. (Introduced by Anson Hoyt.)

From the Department of Experimental Medicine, University of Southern California School of Medicine, and the Laboratories of the Los Angeles County General Hospital, Los Angeles, Calif.

Anaphylaxis may be defined as an acute and frequently damaging reaction of a sensitized animal to the specific antigen used in sensitization. This phenomenon has been studied in many animals but only recently has it been described adequately in mice.[1] The experiments cited below not only confirm that mice are susceptible to anaphylactic shock but also indicate that they are refractory to histamine shock.

White mice of an inbred *Olitsky* strain varying in age from 2 to 6 months were sensitized by intraperitoneal injection of 1 ml of egg white (diluted 1:4 with normal saline) every second day to a total of 4 ml, following the procedure of Weiser, Golub and Hamre.[1] Twelve days after the last sensitizing injection anaphylactic shock was produced by injection of 1 ml of the antigen into the tail vein.

The anaphylactic shock syndrome in the mouse was characterized by sluggishness, wobbling gait, dyspnea, cyanosis, paralysis of extremities, loss of sphincter control,

---

* These studies were aided by the Mayer Fund of the Department of Experimental Medicine, University of Southern California School of Medicine.

[1] Weiser, R. S., Golub, O. J., and Hamre, D. M., *J. Inf. Dis.*, 1941, **68**, 97.

struggling convulsions and death. These symptoms never appeared in less than 5 minutes and never terminated in death in less than 11 minutes, nor in more than 1 hour.

Of 53 sensitized animals given a shocking dose of the antigen, 49 showed some anaphylactic reaction and of these 14 died. These animals were tested in groups of 14, 7, 10 and 22 with death rates ranging from 20 to 28%. A minimal reaction was observed in only one of 20 nonsensitized controls which were similarly inoculated with 1 ml of the antigen.

Thirty-five mice were inoculated intravenously with 1 ml of a 1% solution of histamine diphosphate in distilled water (about 400 mg/kg body weight). None of these animals developed clinical evidence of reaction, but one was found dead of unknown cause one hour after injection. It appears questionable whether this fatality was due to histamine shock, and in any case it may be concluded that the mice used in these experiments were essentially refractory to histamine. These conclusions are not jeopardized by earlier reports[2,3] in which deaths that were attributed to histamine occurred instantaneously on injection and were, therefore, probably embolic. Furthermore, the absence of controls and the small number of animals used tend to invalidate those earlier findings.

Twenty-five mice sensitized to egg white were given intravenously 1 ml of the antigen and 5 mg of histamine in 0.25 ml of distilled water. Half the animals were injected with the egg white first, followed within 5 minutes by the histamine, this order being reversed with the others. Results in both groups did not differ significantly from those observed in the previous experiment where antigen alone was used as a shocking agent.

The results of these experiments are summarized in Table I.

TABLE I.

Material injected and animals used	No. of animals	% showing at least minimal shock	% dying
Egg white in sensitized mice	53	92	26
Egg white in non-sensitized mice	20	5	0
Histamine in non-sensitized mice	34*	0	0
Egg white and histamine in sensitized mice	25	96	12

* One mouse found dead of unknown cause one hour after injection not included in this summary.

This failure of mice to react to histamine while showing susceptibility to anaphylactic shock could not be explained by an antihistamine substance in mouse serum. Seven guinea pigs were injected intravenously with 0.5 mg of histamine diphosphate in normal saline (an experimentally determined M.L.D.), and 6 others with a similar dose of histamine in a 1:2 dilution of normal mouse serum, this mixture having stood at room temperature for 10 to 60 minutes before administration. The frequency and severity of histamine shock in these 2 groups appeared equal. Mouse serum alone had no apparent effect when injected intravenously into 3 guinea pigs.

Similarly, the difference between mice and guinea pigs in susceptibility to histamine did not appear to be explainable on the basis of an histamine-activating factor in guinea pig serum, for guinea pig serum mixed in equal parts with a 1% histamine diphosphate solution and injected intravenously into 10 mice produced no reaction.

Summary. In this series of experiments it has been suggested that the mouse is highly resistant to histamine shock although regularly susceptible to anaphylaxis; that histamine administered either immediately before or after the antigen failed to potentiate the reaction; and that mouse serum *in vitro* failed to detoxify histamine for the guinea pig while guinea pig serum *in vitro* failed to make histamine toxic for the mouse. Further studies of the pathogenesis of anaphylactic shock in the mouse are in progress.

It is therefore concluded that there is no evidence at the present time that histamine plays a direct or significant role in anaphylactic shock in the mouse.

---

[2] Voegtlin, C., and Dyer, H. A., *J. Pharm. and Exp. Therap.*, 1924, **24**, 101.

[3] Schmidt, G. W., and Staehlin, A., *Z. f. Immunitatsforsch.*, 1929, **60**, 222.

15634 P

Influence of Sodium Benzoate by Ingestion upon Urinary Excretion of Streptomycin.

JOHN A. KOLMER, AMEDEO BONDI, JR., HALSAY F. WARNER, AND CATHERINE DIETZ.

From the Department of Bacteriology and Immunology, Temple University School of Medicine, and the Research Institute of Cutaneous Medicine, Philadelphia, Pa.

Bronfenbrenner and Favour[1] have reported that the oral administration of benzoic acid, which is converted by the liver into hippuric acid, reduces the degree and prolongs the duration of the urinary excretion of penicillin, administered by intermittent intramuscular injection, with the result that the serum levels of the compound are increased and longer sustained. Bohls, Cook and Potter[2] have also reported that intramuscular injections of alum-precipitated penicillin along with the oral administration of sodium benzoate resulted in high and prolonged serum levels due in part to the fact that the penicillin was more slowly excreted. Spaulding, Bondi and Early[3] have likewise observed that after the oral administration of single doses of 100,000 units of alum-precipitated penicillin along with 1.2 g of sodium benzoate, the serum levels were slightly higher and longer sustained than after the administration of penicillin alone; similar and somewhat better results were observed following the oral administration of single doses of 100,000 units of aluminum penicillin (not alum-precipitated penicillin) along with 2.4 g of sodium benzoate. These results have been ascribed to the effects of hippuric acid, resulting from the conjugation of sodium benzoate, in the selective excretion of penicillin by the tubules of the kidneys.

As in the case of penicillin, it is highly probable that the excretion of streptomycin by the kidneys is not only by filtration through the glomeruli but also by way of the renal tubules. In this connection, however, it does not appear that streptomycin is excreted by the kidneys in as high a degree as penicillin, with the suggestion that the compound is longer retained or undergoes more inactivation in the body following parenteral administration. Be that as it may, the effects of sodium benzoate upon the urinary excretion of streptomycin have not been previously reported upon and the results of experiments herein described indicate that the oral administration of sodium benzoate does not appear to reduce the urinary excretion of streptomycin with higher and longer sustained serum levels of the compound, as has been observed with penicillin.

In these experiments, single doses of streptomycin* dissolved in 2 cc of sterile saline solution were administered by intramuscular injection to adults of approximately the same body weight. At intervals of 1, 2, 3, 4, 6 and 24 hours thereafter blood and urine were collected for assay purposes, each specimen of urine being measured and the total excretion of streptomycin calculated on the basis of units per cc. Some individuals were given streptomycin alone, as controls, while others were given streptomycin along with sodium benzoate in tablets by ingestion. The first dose of the latter was administered 4 hours before the intramuscular injection of streptomycin, followed by 5 additional doses at intervals of 4 hours. All assays were conducted according to the method of Stebbins and Robinson[4] employing *Staphylococcus aureus* (S M strain).

The serum levels in 3 adult subjects (No. 1, 2 and 3) receiving single intramuscular injections of streptomycin alone in dose of 0.25 g (250,000 units) were 1.5, 3.0 and 2.5 units per cc, respectively, at the end of 6 hours while the total urinary excretion of the compound over a period of 24 hours following its administration varied from 22 to 48% of the amount administered.

---

[1] Bronfenbrenner, J., and Favour, C. B., *Science*, 1945, **101**, 673.

[2] Bohls, S. W., Cook, E. B. M., and Potter, R. T., *J. Ven. Dis. Inform.*, 1946, **27**, 69.

[3] Spaulding, E. H., Bondi, A., Jr., and Early, E., unpublished, personal communication.

* Streptomycin sulfate kindly supplied by the Abbott Laboratories, North Chicago, Ill.

[4] Stebbins, R. B., and Robinson, H. J., PROC. SOC. EXP. BIOL. AND MED., 1945, **59**, 255.

The serum levels in 3 additional adult subjects (No. 4, 5 and 6) following single intramuscular injections of 0.25 g (250,000 units) of streptomycin along with the oral administration of sodium benzoate in dose of 10 grains (0.65 g) for 6 doses totalling 60 grains (3.9 g) in subject No. 4 and 20 grains (1.3 g) for 6 doses totalling 120 grains (7.8 g) in subjects No. 5 and 6 were practically the same at the end of 6 hours, being 1.0, 2.5 and 2.5 units per cc, respectively. Furthermore, the total urinary excretion of streptomycin in these subjects was actually higher than observed in the 3 given streptomycin alone, having varied from 36 to as much as 75% of the dose of streptomycin administered.

In an additional experiment, 0.5 g (500,000 units) of streptomycin were given to each of 2 adult subjects (No. 7 and 8) by intramuscular injection. One of these (No. 7) served as a control while the second (No. 8) was given 20 grains (1.3 g) of sodium benzoate every 4 hours totalling 120 grains (7.8 g) in the period of 24 hours. The results were similar in that the serum level at the end of 6 hours (2 units per cc) in subject No. 8 receiving sodium benzoate was practically the same (2.5 units per cc) as observed in the control. In this experiment, however, while the control (No. 7) showed a total urinary excretion of 44% of the dose of streptomycin administered, subject No. 8 receiving sodium benzoate showed a total urinary excretion of only 30% of the dose of streptomycin injected, while the serum levels at the end of 2, 3 and 4 hours after injection were slightly higher than were observed in the control.

Summary. The results indicate, therefore, that the oral administration of sodium benzoate in tablets in dose of 10 to 20 grains (0.65 to 1.3 g) every 4 hours for 6 doses totalling 60 to 120 grains (3.9 to 7.8 g) along with single intramuscular injections of 0.25 and 0.5 g (250,000 and 500,000 units) of streptomycin does not appear to reduce materially the urinary excretion of streptomycin, and does not enhance or prolong the maintenance of the serum levels of the compound. It is to be emphasized, however, that these preliminary results are inconclusive and, in view of the clinical importance of the subject in relation to the administration of streptomycin, additional investigations are being conducted.

15635 P

Cultivation of African Sleeping Sickness Trypanosomes on Improved, Simple, Cell-Free Medium.*

DAVID WEINMAN.

From the School of Public Health, Faculty of Medicine, Columbia University.

Progress in the investigation of many important aspects of African trypanosomiasis should be appreciably facilitated, it would seem, were it possible to grow the causative organism readily and in large quantities *in vitro*. Following the cultivation of the closely related *Trypanosoma brucei* by Novy and McNeal,[1] which acted as a notable stimulus, possible cultural methods for the human trypanosomes were investigated by Behrens,[2] Ponselle,[3] von Razgha,[4] Reichenow[5] and others. Since none of the methods devised was completely satisfactory, improvements were sought by Brutsaert and Henrard[6] and, more recently, by Weinman,[7] with a semisolid agar. This last permits of indefinite serial transfers and is satisfactory for certain, but not for all purposes; accordingly, experiments were continued until the medium herein reported was developed.

---

* It is a pleasure to acknowledge the aid received from Harvard University and The American Foundation for Tropical Medicine, Inc., joint sponsors of the 1944 Harvard Expedition to Liberia, during which the author obtained the strains of trypanosomes studied.

[1] Novy, F. G., and McNeal, W. J., *J. Inf. Dis.*, 1904, **4**, 1.

[2] Behrens, C. A., *J. Inf. Dis.*, 1914, **15**, 24.

[3] Ponselle, A., *C. R. Acad. Sci.*, 1924, **178**, 1219.

[4] von Razgha, A., *Z. f. Parasitenkunde*, 1929, **2**, 55.

[5] Reichenow, E., *Z. f. Parasitenkunde*, 1932, **4**, 784.

This medium, which is suitable for the cultivation of both *T. gambiense* and *T. rhodesiense,* appears to have advantages over those previously described. Its favorable features are that growth is sufficiently heavy so that the trypanosomes can be obtained in large quantities and in almost any concentration desired, that the organisms thus harvested may be washed free of culture medium, that the growth may be initiated from small inocula of blood, and that the medium is relatively simple, easy to prepare, and stable, so that large quantities may be made prior to use and stored.

The medium is essentially a solid blood agar, containing inactivated, citrated, human plasma. The formula is as follows:

1. Base (autoclaved portion)

Nutrient agar (Difco 1.5%,† pH 7.3)	31 g
Distilled water, to make	1000 cc

For approximately 100 cc of medium, take 75 cc of the base and complete as follows:

2. Nonautoclaved portion

Human citrated plasma, inactivated‡	12.5 cc	
Human red cells	12.5 cc	
		25 cc
Base		75 cc

The medium can be dispensed in Kolle flasks or slanted in test tubes, and if stoppered with rubber corks or cotton covered with Parafilm to retard drying, may be stored in the refrigerator (5-8°C) for long periods.

Inoculated flasks are also stoppered to prevent evaporation and incubated at room temperature. Growth can be detected macroscopically by the development of small, rounded, colorless, transparent, slightly raised, glistening, moist-appearing areas, spotting the surface of the medium. These are colonies. They adhere to the surface of the agar, measure usually about 1-2 mm in diameter, more rarely up to 4 mm, do not hemolyze nor discolor the medium, and are composed of hundreds, or even thousands, of motile trypanosomes.

The numbers of trypanosomes inoculated determine the time required for the cultures to develop. If blood from the donor animal is diluted to contain 1,000 to 10,000 trypanosomes, growth is detectable in 5 days, and is heavy in 10; whereas if the inoculum contains 10 to 100 organisms, the cultures are often negative at 5 and positive at 10 days. Exceptionally, 3 or 4 weeks may be required before development is evident.

The period of heavy growth is usually maintained for about 10 days, then the trypanosomes gradually become less numerous, are quite scanty at the end of 35 to 40 days, but may persist for more than 60 days. Maintenance of the cultures by serial transfer presents thus no special problem. It becomes even simpler if low temperature facilities are available, Weinman and McAllister[8] having shown that cultures may be frozen and stored for long periods at —70°C.

A single harvest from one flask at or near the peak growth yields 10,000,000 to 15,000,000 trypanosomes. The organisms can be washed off in Tyrode's solution, and then concentrated by centrifuging. Washing and centrifuging may be repeated, so that ultimately one obtains a closely packed sediment consisting of trypanosomes surmounted by a water-clear liquid. As any number of

---

[6] Brutsaert, P., and Henrard, C., *C. R. Soc. Biol.*, 1938, **127**, 1469.

[7] Weinman, D., PROC. SOC. EXP. BIOL. AND MED., 1944, **55**, 82.

† Manufacturer's formula for Difco nutrient agar 1.5%:

Beef extract	3 g
Bacto peptone	5 g
Sodium chloride	8 g
Agar	15 g
	31 g

To be dissolved in 1 L. distilled water.

‡ At 56°C for 30 min.

[8] Weinman, D., and McAllister, J., *Am. J. Hyg.*, in press.

flasks may be harvested at one time, and the yield combined, the number and concentration of trypanosomes can be adjusted within wide limits.

The multiplication in culture is massive and from an inoculum of 100 trypanosomes, some 30,000,000 may be obtained in a few weeks. This suggests that indefinite serial transfer will be possible on this medium, as it was on an earlier one,[7] which the author now considers inferior. However, since the strains have been maintained through only 10 generations *in vitro* the matter is not yet certain. Studies on the morphology and properties of the cultural trypanosomes will be reported shortly.

Conclusion. A blood-agar cell-free medium of relatively simple composition is described, which is suitable for the isolation and growth of both *Trypanosoma gambiense* and *Trypanosoma rhodesiense,* and appears to have advantages over others previously described.

15636

Effect of Diethylstilbestrol upon the Systolic Blood Pressure of Normal Rats.

Henry C. Hill, Jr. (Introduced by K. S. Grimson.)

From the Department of Surgery, Duke University School of Medicine, Durham, N.C.

The effect of diethystilbestrol upon the systolic blood pressure of rats has been variously reported. Grollman, Harrison and Williams[1] described hypertension during continuous oral administration in normal rats. Leathem and Drill[2] employed intramuscular injection in normal and in hypophysectomized rats and observed hypertension. Recently Oster[3] used oral administration in castrated rats and likewise demonstrated upward trends of blood pressures. Matthews, Emery, and Weygandt,[4] on the other hand, failed to obtain significant trends toward elevation of systolic blood pressure in normal or in castrate rats after prolonged oral administration during periods of treatment up to 100 days. These varied reports prompted a reinvestigation of the effect of diethylstilbestrol upon the blood pressure of normal rats. A preliminary appraisal of the present findings has been reported.[5]

Method. Thirty normal adult male and female albino rats of the Vanderbilt strain weighing between 180 and 360 g were used. They were maintained on Purina Dog Chow and water throughout the experiment. Systolic blood pressure was determined without anesthesia by the indirect tail plethysmographic method of Williams, Harrison, and Grollman[6] except that a 26 mm sphygmomanometer cuff, as recommended by Kempf and Page,[7] was employed. Water introduced into the plethysmograph was heated to body temperature. Systolic blood pressure was followed in 3 groups of rats:

I. Five male and 5 female rats received daily intramuscular injections of 1.0 mg of diethylstilbestrol in 0.2 cc of cottonseed oil for 24 successive days.

II. Five male and 5 female rats received daily injections of 0.2 cc of cottonseed oil for 24 days.

III. Five male and 5 female rats received

---

[1] Grollman, A., Harrison, T. R., and Williams, J. R., Jr., *J. Pharmacol. and Exp. Therap.*, 1940, **69**, 149.

[2] Leathem, J. H., and Drill, V. A., *Am. J. Physiol.*, 1943, **139**, 17.

[3] Oster, K. A., *Exp. Med. and Surg.*, 1946, **4**, 20.

[4] Matthews, C. S., Emery, F. E., and Weygandt, P. L., *Endocrinology*, 1943, **33**, 177.

[5] Hill, H. C., Jr., *Fed. Proc.*, 1946, **5**, 46.

[6] Williams, J. R., Jr., Harrison, T. R., and Grollman, A., *J. Clin. Invest.*, 1939, **18**, 373.

[7] Kempf, G. F., and Page, I. H., *J. Lab. and Clin. Med.*, 1942, **27**, 1192.

TABLE I.
Systolic Blood Pressures of Treated and Untreated Rats for 24 Days.

Group	Substance injected	No. of rats	Sex	Range of B.P. mm Hg.	Mean B.P. mm Hg.
I	Diethylstilbestrol	5	♂	80-128	110.8
	”	5	♀	80-135	116.5
II	Cottonseed oil	5	♂	82-119	104.2
	”	5	♀	72-128	107.3
III	Untreated	5	♂	90-128	111.4
	”	5	♀	80-125	107.5

no treatment for the same period of time and served as controls.

Systolic blood pressures were determined daily for 3 to 6 days before injections and then daily throughout the injection period and the week following treatment. Subsequent readings were obtained at intervals of several days up to the 78th day to determine whether there might be delayed effects.

Results. The effect upon blood pressure of I diethylstilbestrol in cottonseed oil, II cottonseed oil and III control conditions in both male and female rats is presented in Table I. It is evident that although there was some variation both in the range and in the average of the blood pressures, there was no definite hypertension in any group. The blood pressure of the individual rats of each group also did not show a definite hypertension. The greatest variation of blood

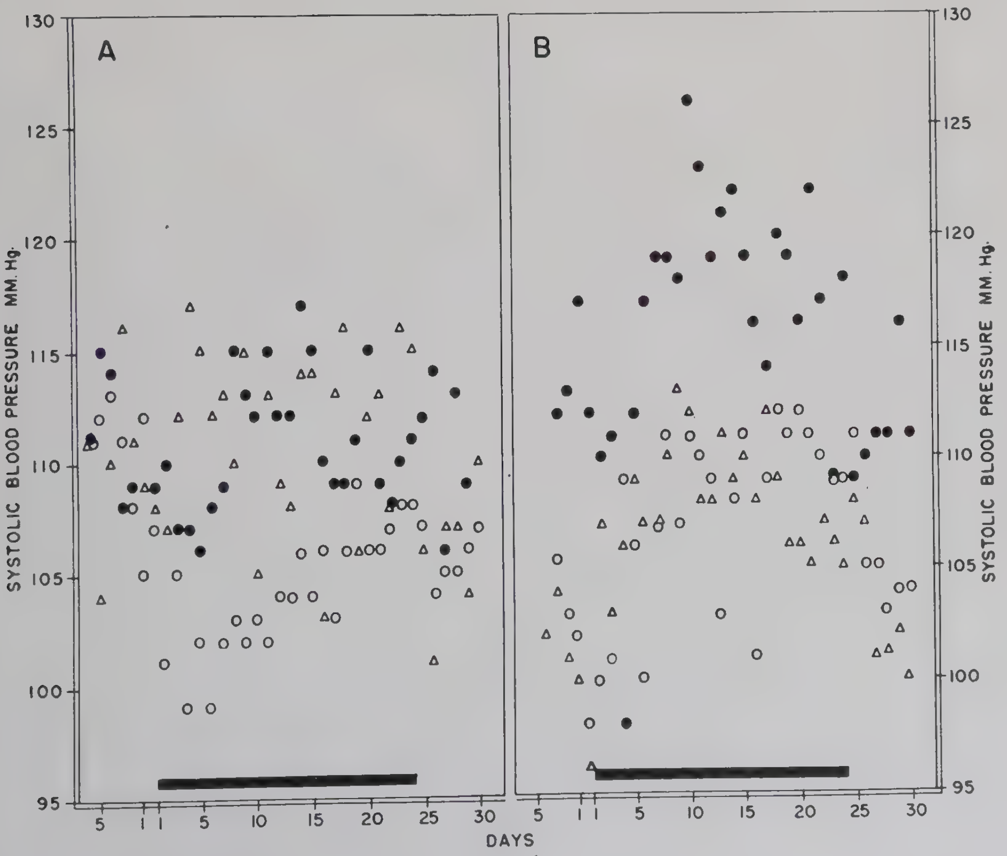


FIG. 1.

pressure of an individual male rat during diethylstilbestrol injection was 11 mm Hg. above the preinjection level and of a female rat 19 mm Hg. These variations are within the normal range for the rat.

There was, however, a slight difference between the blood pressures of the male and female groups. This is illustrated in Fig. 1, in which the blood pressures taken each day on the 5 animals in each group are averaged and plotted. The males are plotted under (A), and the females under (B). It is evident that the male rats receiving cottonseed oil had slightly lower blood pressures than the control rats or those receiving diethylstilbestrol. It is also evident that the blood pressures of the female rats receiving diethylstilbestrol are somewhat higher than the control or cottonseed oil-treated groups. The highest blood pressure readings were observed about the 10th day. Blood pressures obtained at intervals of several days and continued up to 54 days after the period of injection revealed no further change.

Comment. These experiments have yielded results that are in accord with the report of Matthews, Emery and Weygandt[4] that hypertensive levels of blood pressure do not occur in rats during continuous treatment with diethylstilbestrol. However, upward trends of blood pressure, not observed by these investigators, were observed in the present experiments. Hypertension of the degree reported by others[1-3] was not observed. Cottonseed oil when used alone slightly reduced the blood pressure of male rats, but did not affect the pressures of female rats. It, therefore, does not explain the slight elevation observed in the female rats treated by diethylstilbestrol in cottonseed oil.

Summary. Diethylstilbestrol did not induce hypertension in normal rats during 24 days of continuous intramuscular injection. Moderate but transient elevations of systolic blood pressure occurred in female but not in male rats.

15637

Some Cross-Circulation Experiments with Di-β-Chlorethyl Sulfide Poisoned Dogs.

SIMON BLACK AND JOHN F. THOMSON. (Introduced by E. M. K. Geiling.)

From the Toxicity Laboratory and the Department of Pharmacology, University of Chicago.*

Death of animals caused by di-β-chlorethyl sulfide (mustard gas) has been considered by some workers[1] to be due to the liberation from poisoned tissues of a "secondary toxin." We have attempted to test this hypothesis by cross-circulation experiments with dogs.

When cross-circulation between a poisoned and an unpoisoned dog was begun after the original poison had disappeared from the blood of the first member, no symptoms of toxic action were evident in the second animal during a subsequent observation period of 15 days. The dose used was about 30 times the LD_{50} dose.

Experimental. Animals. Mongrel dogs of both sexes weighing 8-10 kg were used.

Cross-circulation. The animals were anesthetized with 30 mg/kg of nembutal intraperitoneally, and given additional small doses intravenously as required during the experiment. The left external jugular vein and left common carotid artery, of one dog, and the corresponding vessels on the right side of the other, were exposed. The 2 animals were then strapped together so that their exposed vessels could be conveniently joined. The distal end of the exposed portion of each vessel was ligated and the proximal end clamped with a bull-dog clamp. The ex-

---

* This work was done under contract with the Medical Division of the Chemical Warfare Service.

[1] Lewis, T. L., and Grant, R. T., *Heart*, 1924, **11**, 209.

TABLE I.
Hematocrit and Plasma Glucose Levels in Blood of Dogs in Cross-Circulation Experiments.
Poisoned animal given 80 mg/kg of mustard[*] intraperitoneally.

				Hematocrit (%)					Plasma glucose (mg %)				
Exp. No.	Survival time of poisoned animal[†] Hr. Min.	Time after poisoning at which cross-circulation began Hr. Min.	Duration of cross-circulation Hr. Min.	Poisoned animal: initial value	Poisoned animal: at beginning of cross-circulation	Unpoisoned animal: at beginning of cross-circulation	Poisoned animal: at termination of cross-circulation	Unpoisoned animal: at termination of cross-circulation	Poisoned animal: initial value	Poisoned animal: at beginning of cross-circulation	Unpoisoned animal: at beginning of cross-circulation	Poisoned animal: at termination of cross-circulation	Unpoisoned animal: at termination of cross-circulation
1	4 : 15	1 : 00	2 : 00	36	49	40	56	56	106	123	130	196	192
2	2 : 10	0 : 35	0 : 45	39	48	38	61	61	115	146	98	230	138
3	4 : 10	0 : 30	3 : 00	47	53	32	60	59	135	232	112	228	249

[*] Given as 10% solution in propylene glycol.

[†] The unpoisoned animals survived beyond a 15-day observation period.

posed artery of each animal was then joined to the vein of the other by means of U-shaped Pyrex glass cannulae with inside diameter of about 3 mm. These cannulae were thoroughly cleaned and dried, and filled with heavy mineral oil before insertion into the blood vessels in order to minimize the danger of intracannular clotting. When it was desired to begin cross-circulation the bull-dog clamps were removed from the vessels. When the experiment was to be terminated the vessels were ligated, the cannulae removed, and the incision closed with wound clips.

The rapidity of exchange of blood by a pair of cross-circulating animals was indicated by a test with Evans Blue dye. A solution of this dye was injected intravenously (fore leg) into one member of a cross-circulating pair one hour after cross-circulation was begun. Two minutes after the injection the concentration of the dye in the venous plasma of the second dog was equal to that in the plasma of the first one.

Results. Injection of poison after cross-circulation was begun. In one experiment a 100 mg/kg dose of mustard was injected intraperitoneally into one member of a cross-circulating pair, and the cross-circulation terminated 30 minutes later. The poisoned dog died within 3 hours. The second dog died within 4 days. Pathological findings[†] in the second dog were typical of severe intravenous mustard poisoning and included widespread hemorrhages of the lungs, pulmonary edema, gangrene of the right lung, and splanchnic hyperemia. The thymus was markedly atrophied.

Injection of poison before beginning cross-circulation. Several experiments were done in which cross-circulation between 2 animals was not begun until 30-60 minutes after one member of a pair was given an intraperitoneal dose of 80 mg/kg of mustard. In all cases the hematocrit values rapidly became identical, or nearly identical, in the 2 animals. The plasma glucose levels also usually rose to approximately the same value (Table I). Despite this evidence of a large exchange of blood between the cross-circulating animals, and the fact that all poisoned members died within 5 hours of poisoning, all of the unpoisoned members survived the experiment. There was no evidence of toxic action, such as loss of appetite or excessive thirst in the unpoisoned members of the cross-circulating pairs.

[†] Autopsy was performed by Dr. C. C. Lushbaugh.

Discussion. The results of these experiments indicate that when a large dose of mustard is given intraperitoneally it rapidly enters the blood stream in considerable quantity. Within 30 minutes, however, it has disappeared from the circulation. This confirms chemical determinations of mustard in the blood of poisoned dogs.[2]

The survival of the unpoisoned members of cross-circulated pairs when cross-circulation began 30-60 minutes after poisoning indicates that no large amount of any "secondary toxin" was present in the circulation after the original poison had disappeared from the blood. It should be emphasized that the dose of the poison was many times larger (80-100 mg/kg) than the LD_{50} dose. If production of a "secondary toxin" was proportional to the dose of the original agent, a relatively small exchange of blood between a poisoned and an unpoisoned animal should have caused death in the second animal. We must conclude, therefore, that death by acute mustard poisoning does not result from liberation of a "secondary toxin" which is freely diffusible into the blood stream.

When smaller doses of the poison are used death is delayed for several days. The cross-circulation technic is not suited to determining whether a "secondary toxin" is involved in such cases.

Summary. 1. A large intraperitoneal dose of di-β-chlorethyl sulfide was given one member of a cross-circulating pair of dogs. Death was produced in both animals, though it was much delayed in the second member. 2. Cross-circulation between 2 dogs was begun 30-60 minutes after poisoning one with a large intraperitoneal dose of mustard. In no case was death produced in the second, unpoisoned dog. 3. It is concluded that a "secondary toxin" is not responsible for death in acute mustard poisoning.

---

[2] Thomson, J. F., unpublished.

15638 P

Effect of Activity on the Visco-Elasticity of Normal and Iodoacetate Muscles.

ALEXANDER SANDOW AND MANFRED BRUST. (Introduced by Harry A. Charipper.)

From the Department of Biology, Washington Square College of Arts and Science, New York University.

Gasser and Hill's studies[1] on the visco-elasticity of muscle by the damped vibrating spring method have been questioned in subsequent work.[2-4] The present research employs the spring technic, but criticisms of it have been largely overcome by eliminating slack of the muscle at all times, by holding constant the initial length of any muscle subjected to a series of tests, and by limiting visco-elastic comparisons to either states of contraction or rest as modified by previous activity.

Excised, Ringer's-equilibrated *Rana pipiens* sartorii were used throughout. The muscles (average excised length, 34.8 mm) were stretched 15% when attached to the undeflected spring, and a further 15% stretch occurred with the spring maximally deflected.

In the work reported below, each plotted value represents the average behavior of at least 3 different muscles, and, in general, the mean deviation for the viscosity coefficient values is about ±6%, and for the elasticity coefficient values, about ±12%.

---

[1] Gasser, H. S., and Hill, A. V., *Proc. Roy. Soc., B*, 1924, **96**, 398.

[2] Hogben, L. T., and Pinhey, K. F., *Br. J. Exp. Biol.*, 1926, **4**, 196.

[3] Steinhausen, W., *Pflüg. Arch. f. d. ges. Physiol.*, 1926, **212**, 31.

[4] Schoepfle, G. M., and Gilson, A. S., Jr., *J. Cell. and Comp. Physiol.*, 1946, **27**, 105.

Fig. 1 shows the progressive relative effects of a series of 5 sec. maximum isometric tetani of the normal muscle (the spring acting as the isometric lever) when the visco-elastic determinations were made during 25-sec. rest periods interposed between the tetani. Apart from the initial rise in the viscosity coefficient, the progressive effect of the activity is to cause a general reduction in both the viscosity and elasticity of the resting, gradually fatiguing, muscle.

Fig. 2 presents the changes in the visco-elastic behavior when the determinations are made during each of the 5-sec. tetani of a tetanus series of a normal muscle. Here the viscosity and the elasticity become progressively reduced in the muscle as it becomes more and more fatigued. Thus, whatever the values of the viscosity and the elasticity coefficients in the normal muscle, initially at rest or during the first tetanus, the effect of the development of fatigue is to reduce these coefficients within each of the series of successive contracting or resting states, although, as comparison of Fig. 1 and 2 demonstrates, the course of the changes in the resting series is not paralleled in detail by that in the contracting one.

Fig. 3 and 4 summarize the results of experiments essentially like those above, except that the muscles involved had been treated with 1:80,000 iodoacetic acid (IAA). The intratetanic visco-elastic changes are similar to those of the normal muscles, though they develop with activity more rapidly. But in the IAA intertetanic series, viscosity and elasticity, after initially falling, as in the normal muscles, then show behavior different from that of the unpoisoned tissue in the sharp increases in both coefficients which occur after the first 10 to 15 sec. of activity, *i.e.* the period during which the IAA rigor develops.

The interpretation of these results is difficult for it is not even certain that the observed changes are attributable to alterations in the contractile material, since, as

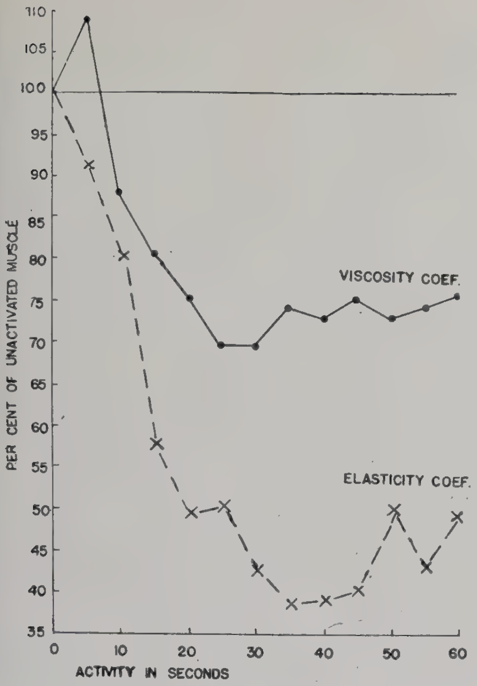


Fig. 1.
Normal muscle: intertetanic changes.

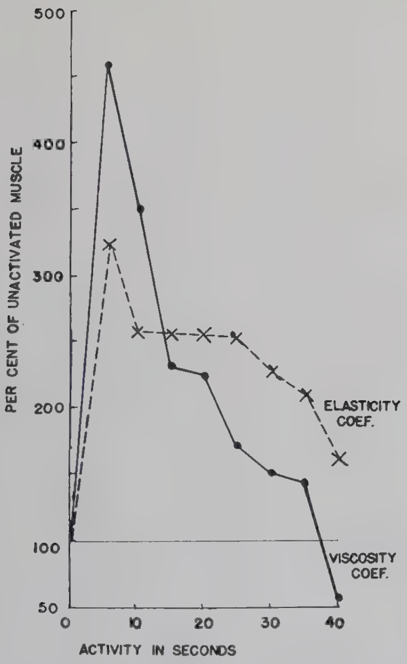


Fig. 2.
Normal muscle: intratetanic changes.

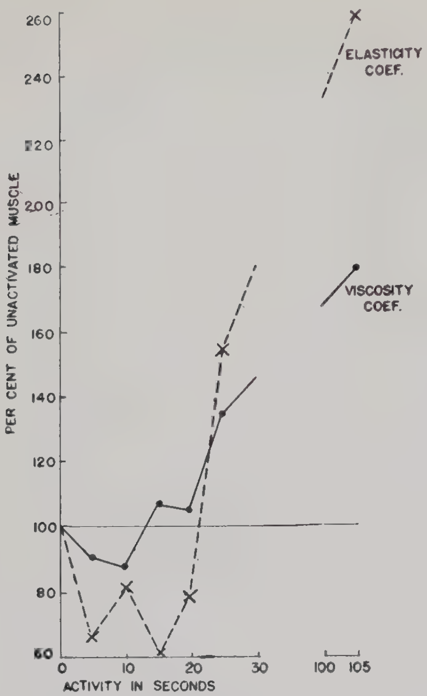


FIG. 3.
IAA muscle: intertetanic changes.

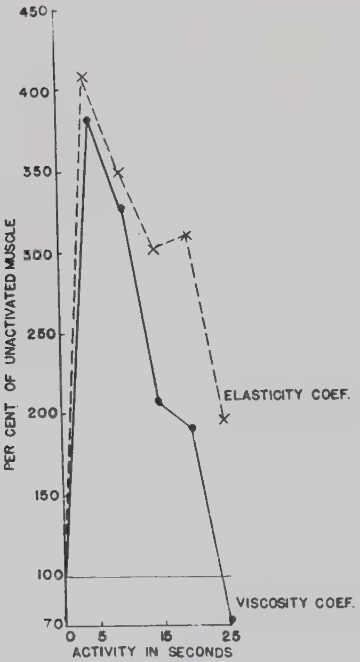


FIG. 4.
IAA muscle: intratetanic changes.

indicated by recent work,[4,5] important elastic properties are associated with the sarcolemma of the muscle fiber. However, the sarcolemma is quite inert and its elastic behavior should remain constant as a given muscle is altered, provided, as is the case in our experiments, the initial muscle length is held constant.[6] It is noteworthy that our method can reflect known changes in the myosin, for muscles treated with 2.5 M urea show a marked reduction in viscosity (and, incidentally, elasticity also)[7] like that found[8,9] after similar treatment of myosin solutions. Furthermore, our intertetanic results for the IAA muscle especially indicate that the observed visco-elastic changes are determined by modifications in the state of the contractile material, since these alterations show a certain correlation with the onset of the rigor that activity causes to appear in the contractile system poisoned with IAA.

Thus, it is possible that our results in general refer to changes in the properties of the contractile system under the influence of progressively developing fatigue. The recent work of the Engelhardt[10] and of the Szent-Györgyi[11] schools on the mechanical and enzymatic properties of muscle proteins shows that these systems, in consequence of their involvement in a variety of mechano-chemical coupling reactions, undergo marked visco-elastic changes. We cannot present here a detailed analysis of the relation between these changes and our observations. It is suggested, however, that evidence such as ours may be used to test the validity of contraction schemes based on the behavior of protein systems extracted from muscle.

---

[5] Ramsey, R. W., and Street, S., *J. Cell. and Comp. Physiol.*, 1940, **15**, 11.

[6] Ramsey, R. W., personal communication.

[7] Brust, M., *Biol. Bull.*, 1946, **91**, 221.

[8] Edsall, J. T., and Mehl, J. W., *J. Biol. Chem.*, 1940, **133**, 409.

[9] Mommaerts, W. F. H. M., *Naturwiss.*, 1944, **32**, 78.

[10] Engelhardt, V. A., *Yale J. Biol. and Med.*, 1942, **15**, 21.

[11] Szent-Györgyi, A., *Acta Physiol. Scand.*, 1945, **9**, Suppl. XXV.

15639

Effect of Exogenous Superinfection on Naturally Acquired Bovine Tuberculosis in Inbred Rabbits.*

Max B. Lurie and Joyce Becker.

From the Henry Phipps Institute, University of Pennsylvania.

Considerable evidence[1] suggests that continued exposure to exogenous contagion is an important factor in the origin of active tuberculosis in tuberculin-positive individuals. However, little is known of the effect of this continued exposure on an already existing active disease. The purpose of the present report is an attempt to answer the above question.

In a previous paper[2] it was shown that increasing concentrations of tubercle bacilli in the enviromental air of the highly inbred, genetically resistant rabbit family A, increases proportionally the incidence of the acquired disease, accelerates its onset, intensifies its dissemination and shortens its duration. In the inbred families C and F, of low resistance, an increase in the intensity of the contagion also resulted in an increase in the incidence of the acquired disease; however, its character was uniformly rapidly disseminating and of short duration whether acquired under conditions of high or low contagion. Since the disease originated as a single pulmonary focus under all conditions, the acceleration of the tuberculous process in the resistant family with increasing intensities of contagion could not be readily ascribed to a larger number of initially inhaled particles capable of producing pulmonary foci under higher intensities. Nor does it seem likely that the number of bacilli contained in the individual infectious particles in the air varies with the intensity of the contagion under which they arise. However, it is probable that after the disease had taken root, additional exogenous infective particles penetrate the lung more frequently under high than under low intensities of contagion. Therefore it seemed desirable to determine whether continued intense exposure to the inhalation of tubercle bacilli affects adversely the course of an already engrafted disease in animals of high and low resistance.

Materials and Methods. Accordingly, the following experiment was set up. Litter mate pairs of the families A and C were exposed to tuberculous rabbits, half of which were infected intravenously and half intrarenally[2] with highly virulent bovine tubercle bacilli (Ravenel). The artificially-infected rabbits were in runs on one side of a fine wire-mesh screen. The contacts were in individual cages, with wire-mesh walls in back and in front, on the other side of the screen, and 6 inches from it. The 3-tiered manifold previously described[3] containing both the sources of contagion and the contacts, was separated into 2 equal halves by a wooden partition.[4] To equalize the intensity of the contagion on both halves of the manifold the sources of contagion were interchanged daily between the 2 adjoining rooms. There were 8 sources of contagion and 10 contacts in each tier. As the sources of contagion died from tuberculosis they were replaced by similarly infected rabbits throughout the course of the experiment.

At biweekly intervals all the contacts were tested with tuberculin. Once a month roentgenograms of the chests of the contacts were taken. As soon as a clearly positive tuberculin reaction appeared in a certain contact it was immediately removed from the contagion and was placed in another room free from artificially-infected animals. Its litter mate, on the other hand, was left in contact with the sources of contagion in the manifold even after the appearance of a posi-

---

* Aided by a grant from the Commonwealth Fund.

[1] Chadwick, H. D., and Pope, A. S., *The Modern Attack on Tuberculosis,* The Commonwealth Fund, New York, 1946.

[2] Lurie, M. B., *J. Exp. Med.,* 1944, **79**, 573.

[3] Lurie, M. B., *Am. Rev. Tuberc.,* Suppl., 1941, **44**, 1-125.

[4] Lurie, M. B., *J. Exp. Med.,* 1944, **79**, 559.

TABLE I.

Effect of Exogenous Superinfection on Course of Naturally Acquired Air-Borne Tuberculosis in the Highly Inbred Family C.

Exposed to superinfection						Not exposed to superinfection					
Rabbit No.	Parents	Preallergic period in mos.	Max. inflammation to tuberculin in mm³	Duration of disease in mos.	Presence of tuberculosis in testes	Rabbit No.	Parents	Preallergic period in mos.	Max. inflammation to tuberculin in mm³	Duration of disease in mos.	Presence of tuberculosis in testes
C6-50	C5-52 40	3.3	117	2.3	0	C6-49	C5-52 40	1.9	250	2.6	0
53	52 40	8.2	472	3.8	0	51	52 40	3.5	472	3.7	0
57	52 40	4.0	525	4.9	0	56	52 40	17.5†	None	None	—
59	52 40	4.2	851	4.7	0	58	52 40	10.7	1116	3.8	0
C7-15	C6-11 C5-40	4.9	873	2.6	0	C7-14	C6-11 C5-40	2.3	990	3.6	0
10	C6-11 C5-40	10.5†	None	None	—	18	C6-11 C5-40	11.7	273	5.2	0
9	C6-11 C5-40	2.6	594	1.7*	0	17	C6-11 C5-40	?	256	4.9	0
Avg			572	3.6					571	3.9	

* Died of intercurrent disease and tuberculosis.

† Duration of exposure.

Incidence of disease, 85%.

Average preallergic period, 5.2 mos.

Average maximum tuberculin sensitivity, 565 mm³.

tive tuberculin reaction and remained in contact until its death. The isolated contacts were protected from cross contagion from each other by ultraviolet lamps placed immediately in front and above their cages, as it has been clearly shown that ultraviolet radiation eliminates the contagion of tuberculosis.[4] Thus half of the rabbits were not exposed to exogenous superinfection, while the other half of the litter mates of the same genetic history for the past 6 to 9 inbred generations and of the same environmental experience over a period of 11 years were exposed to continuous, intense exogenous superinfection. All but a few of the litter mate pairs were of the same age and sex.

Results. In Table I, Columns 2 and 8 record the parents of both groups of rabbits of the C family, of low genetic resistance to the disease. It is evident that the genetic history of both was identical. In Columns 5 and 11 are recorded the duration of the disease in these genetically similar rabbits. It is clear that there was no difference in the rate of progression of the disease whether the contact remained in continuous exposure to the contagion or whether it was removed therefrom immediately after the acquisition of a positive tuberculin reaction. In both instances the disease pursued its inexorable, rapid course. Those exposed to superinfection died of tuberculosis within an average of 3.6 months with a survival range of 2.3 to 4.9 months. Those not exposed to superinfection died within an average of 3.9 months with a survival range of 2.6 to 5.2 months. While those removed from contagion survived a little longer, the difference is clearly not significant. That the contagion to which the rabbits were exposed was very high is seen in the fact that 12 of the 14 contacts died with tuberculosis, an incidence of 85%. The rapidity of attack by tuberculosis, or the average interval elapsing between the beginning of exposure and the development of tuberculin sensitivity, was 5.2 months. It is noteworthy that the maximum average sensitivity to tuberculin developed by both groups of rabbits was the same. Continuous exposure to exogenous superinfection did not increase the allergic sensitivity developed during the course of the disease. Columns 6 and 12 show that in none of the 12 rabbits of this genetically susceptible family did tuberculosis of the testicle develop. This is significant since testicular disease nearly always results from tubular spread of medullary renal tuberculosis and is frequently associated with a chronic slowly progressive disease. Thus, whether the contacts were or were not exposed to superinfection, no case of chronic tuberculosis occurred.

In Table II are given the corresponding data for the rabbits of the genetically-resistant family A. Columns 2 and 8 show that the genetic history of the rabbits exposed to superinfection and those not exposed to superinfection was identical in most instances. It is clear from Columns 5 and 11 that the disease in the rabbits of this family was, in general, more chronic than that in the contacts of the C family. The difference, however, is not great. As demonstrated in a previous study[2] a high intensity of contagion tends to overwhelm the natural resistance of this family. Nevertheless, the incidence of the acquired disease was distinctly lower among the A rabbits as compared with those of the C family, an incidence of 73% instead of 85% in the latter. Likewise, the duration of the disease of the A rabbits, both those exposed and those not exposed to superinfection, was longer, an average of 4.6 and 5.4 months as compared with 3.6 and 3.9 months respectively, for the rabbits of low resistance. It is noteworthy that A rabbits tended to acquire tuberculin sensitivity before the C rabbits. The average preallergic period was 4.2 months for the former and 5.2 for the latter. This is in agreement with previous observations, where evidence was presented that the more resistant A rabbits acquire tuberculosis after a shorter interval of natural exposure than the less resistant C family.[2] Again, the intensity of allergic sensitivity of the A rabbits tended to be considerably less than that of the C rabbits, the maximum average sensitivity of the former being 321 mm^3 as compared with 565 mm^3 for the C rabbits. This also confirms previous ob-

TABLE II.

Effect of Exogenous Superinfection on Course of Naturally Acquired Air-Borne Tuberculosis in the Highly Inbred Family A.

Exposed to Superinfection						Not exposed to superinfection					
Rabbit No.	Parents	Preallergic period in mos.	Max. inflammation to tuberculin in mm³	Duration of disease in mos.	Presence of tuberculosis in testes	Rabbit No.	Parents	Preallergic period in mos.	Max. inflammation to tuberculin in mm³	Duration of disease in mos.	Presence of tuberculosis in testes
A8-82	A7-3 A6-27	1.4	64	3.4	0	A8-83	A7-3 A6-27	10.7	405	5.0	+
						81	A7-3 A6-27	5.6	64	1.0*	0
						A7-3	A5-30 A6-2	2.0	75	3.7	0
A9-21	A8-61 60	4.0	460	4.4	0	A9-20	A8-61 60	2.3	375	3.0	+
23	61 60	3.3	492	3.5	0	22	61 60	4.9	500	3.6	+
24	61 60	0.9	343	4.4	0	25	61 60	4.3†	None	None	—
35	61 62	6.0	546	7.3	+	33	61 62	5.1	208	12.0	+
						39	66 62	13.8†	None	None	—
48	59 64	12.2†	None	None	—	47	59 64	12.2†	,,	,,	
Avg				4.6						5.4	

*Rabbit had a single primary pulmonary focus with slight caseation in homolateral hilum node. Died of intercurrent disease.

† Duration of exposure.

Incidence of disease, 73%.

Average preallergic period, 4.2 mos.

Average maximum tuberculin sensitivity, 321 mm³.

servations.[3] Finally, in 5 of the 10 rabbits that died of tuberculosis of this family, there was tubular spread of the disease to the testicles, while none of 11 rabbits of the C family showed a tuberculous orchitis. All these observations show that, while the difference in survival between the A and C rabbits was not marked under this high intensity of contagion, the A rabbits still retained some of the essential responses to the disease characteristic of this family in the past where, under conditions of low contagion, the contrast between the chronicity of the disease in the A family and its fulminating course in the C family was marked.

As to the main question at issue, it is clear that under these conditions of intense contagion there was no significant difference in the course of the disease in the rabbits which remained in continuous exposure to contagion after the acquisition of the disease as compared with those who were removed from contagion after their acquisition of tuberculin sensitivity. Again, as with the C family, the A rabbits not exposed to exogenous reinfection survived somewhat longer, an average of 5.4 months, than those continuously exposed 4.6 months. However, the difference is hardly significant.

Summary and Discussion. Under conditions of natural intense exposure to virulent bovine bacilli, rabbits of high and low genetic resistance to tuberculosis were not benefited by cessation of the exposure after the onset of the disease. The disease pursued its characteristic course whether exogenous superinfection took place or not.

That the course of the disease in the rabbits of low genetic resistance was not affected by the cessation of exposure after the onset of the disease may have been anticipated from previous data[2] where it was shown that in the C family the disease pursued the same fulminating course whether acquired under conditions of high or low intensities of contagion. In the resistant family A interruption of the contagion after the onset of the disease tended to retard somewhat the progression of the disease. However, the difference was slight. It is clear therefore that exogenous superinfection after the acquisition of a positive tuberculin reaction does not explain the intensification of the dissemination of the disease and the shortening of the duration observed in this family, with increasing concentrations of bacilli in their environment. It is possible that the initial inhalation of larger numbers of bacilli which do not at once give rise to visible pulmonary foci under high intensities of contagion may account for the more rapid progression of the disease in this family under these conditions.

It is noteworthy that while the differences between the rabbits of high and low resistance to tuberculosis were greatly diminished by the intense contagion prevalent in these experiments, they were not completely wiped out. As in previous studies the A rabbits tended to acquire tuberculin sensitivity more rapidly than the C rabbits, their allergy was less developed during the course of their disease and the progression of the infection was slower and anatomically more chronic.

It must be emphasized that these data do not warrant the conclusion that in human tuberculosis exogenous superinfection plays no role in the course of an existing disease. In the first place, bovine tuberculosis in rabbits is uniformly fatal, while a large proportion of active human pulmonary disease is arrested. In the second place, the intensity of contagion under which the disease was acquired in this study was so high that 73 to 85% of the exposed rabbits acquired tuberculosis, an incidence which never occurs in human life. Therefore, these experiments are not strictly comparable to those obtaining in the human disease.

15640

Development of Streptomycin Resistance of Gram-Negative Bacilli *In vitro* and During Treatment.*

Roderick Murray, Lawrence Kilham, Clare Wilcox, and Maxwell Finland.

From the Thorndike Memorial Laboratory, the Mallory Institute of Pathology, Boston City Hospital, and the Department of Medicine, Harvard Medical School, Boston, Mass.

In the course of studying a group of cases treated with streptomycin for infections with Gram-negative bacilli, therapeutic failures were found to be associated with the development of extreme resistance to that agent.[1] An attempt was then made to produce similar resistance *in vitro* in some of the streptomycin-sensitive organisms which had been isolated before therapy was started. The strains made resistant *in vitro* were then compared with the original organisms and with the resistant strains isolated from the same patients during or after treatment.

Two series of studies were done, (1) in liquid media by transfer through increasing concentrations of streptomycin in broth and (2) on solid media containing similar concentrations of streptomycin.

Methods. The two types of media used in the present study were: "Brain Heart Infusion" (Difco), broth, pH 7.3, hereafter referred to as broth, and "Heart Infusion Agar" (Difco), pH 7.4 to be referred to as agar. While not the optimum media for streptomycin studies,[2-4] they were highly satisfactory for the purpose at hand.

Streptomycin concentrations were checked for potency against the reference organisms used in this laboratory, namely, strain T of *Klebsiella pneumoniae*, type A, which is sensitive to 0.78 unit of streptomycin. This organism has given consistent and duplicable results. Organisms isolated from patients prior to treatment were subcultured successively on agar plates 4 or more times, by streaking from a single colony on each occasion. Eventually a single such colony was picked off and used to provide the starting culture for both series of tests.

The broth transfer study was carried out by a serial dilution method. A 2-fold concentration series of streptomycin in broth was set up in 10 mm x 110 mm tubes by pipetting 0.5 ml quantities from "master" tubes prepared by serial dilution. The range of each series was chosen according to the anticipated resistance of the organism being studied. The tubes were then inoculated with 0.5 ml of a 10^{-4} dilution of a 24-hour broth culture of this organism bringing the total volume in each tube to 1.0 ml. The inoculum contained approximately 100,000 to 150,000 organisms, the actual number present being followed by pour plates; it also contained 1.0% of defibrinated horse blood to act as an indicator of bacterial growth. This amount of horse blood neither stimulates growth of the reference organisms nor inhibits streptomycin action in this medium with the size of inoculum used. Tubes were incubated and examined for visible evidence of growth after 18 to 24 hours. The lowest concentration of streptomycin in which growth failed to occur, as determined by subculture on streptomycin-free agar plates (or in streptomycin-free broth tubes in the case of *B. proteus*) was read as the minimum inhibiting concentration (M.I.C.). The tube with the highest concentration of streptomycin in which there was good bacterial growth was used as the source culture for a fresh series of tubes. This process was repeated daily, the time per transfer thus being ap-

---

* The streptomycin was provided by the National Research Council from supplies assigned for clinical investigations recommended by the Committee on Chemotherapeutics and Other Agents. This study was aided, in part, by a grant from the U. S. Public Health Service.

[1] Finland, M., et al., *J. A. M. A.*, 1946, **132**, 16.

[2] Wallace, G. I., Rhymer, I., Gibson, O., and Shattuck, M., Proc. Soc. Exp. Biol. and Med., 1945, **60**, 127.

[3] Donovick, R., and Rake, G., Proc. Soc. Exp. Biol. and Med., 1946, **61**, 224.

[4] Hobby, G. L., Lenert, F., and Hyman, B., (Abst.) *J. Bact.*, 1946, **51**, 606.

TABLE I.
Development of Streptomycn Resistance During Treatment and *in Vitro*.

No.	Case	Organism	M.I.C. of organism isolated before treatment, units/ml	Days treatment to isolation of strain of max. resistance	No. of transfers required to achieve max. resistance	
					In streptomycin broth	In streptomycin agar
1	A.P.	*A. aerogenes*	6.3	3	49	29
2	E.F.	” ”	12.5	1	41	12
3	E.F.	” ”	12.5	1	27	—
4	C.H.	*K. pneumoniæ*	12.5	3	44	19
5	C.M.	” ”	6.3	1	40	6
6	W.T.	Paracolon bacillus	25	4	—	29
7	C.M.	*Ps. aeruginosa*	12.5	3	13	5
8	M.K.	*E. coli*	25	1	44	19
9	M.S.	*B. proteus*	50	*	31	*
					Avg 36	Avg 17

* Organism not recovered after treatment was started and not studied on agar.

Note. Resistant strains grew well in broth containing 50 mg (50,000 units) per ml from an inoculum of 100,000 to 150,000 organisms.

M.I.C. = Minimum inhibiting concentration giving no growth on transfer to streptomycin-free broth.

proximately 24 hours. On occasions when it was necessary to miss a day the tubes were refrigerated and no significant changes in the general trend were noted. Concentrations of 2,000, 5,000, 10,000 and 50,000 units per ml were found to be convenient and satisfactory when resistance had been raised to the 1,000-unit level.

In the second series of studies agar plates were prepared containing increasing 2-fold concentrations of streptomycin by mixing 1.0 ml of broth containing the required amount of streptomycin with 9.0 ml of melted agar. These plates, together with a control containing no streptomycin were each streaked with a 2 mm loopful of an undiluted 24-hour broth culture of the organisms. It was possible to accommodate several tests on each plate. The plates were examined for growth after 24 hours incubation and the lowest concentration of streptomycin showing no growth was read as the M.I.C. Material for transfer to the next series was taken directly from the surface of the plate with the highest concentration of streptomycin on which there was good growth. A 2.0 mm loopful of the surface growth was used for each inoculation. Transfers were made daily. Occasionally when a delay was necessary, transfers were made through streptomycin-free plates to avoid contact of the organisms with streptomycin for longer than 24 hours per transfer. It was found impractical to use concentrations of over 6,400 units per ml in agar. In practice all the organisms studied became highly resistant before this value was reached. When it became evident that an organism had become highly resistant, *e.g.*, when improved growth was noted on concentrations which previously supported only poor growth or when there was a sudden rise in resistance, subcultures were made to streptomycin-free broth and the sensitivity was then determined by the usual serial dilution method. The final M.I.C. values reported for the 2 series of experiments were, therefore, all determined by the serial dilution method in broth and so are comparable.

The streptomycin dilutions used in both series of studies were checked daily against the reference organism. This allowed corrections to be applied for loss of potency of stock streptomycin solutions.

Nine strains were studied, as shown in Table I. Eight of them were from 6 patients who later yielded highly resistant but otherwise indistinguishable strains during or after treatment with streptomycin. One strain of *B. proteus* from a case which responded to therapy was included in the broth dilution series, but not with the surface plate series because of its spreading properties. Strain No. 1 was obtained from the blood culture of a patient who died of bacterial endocarditis;

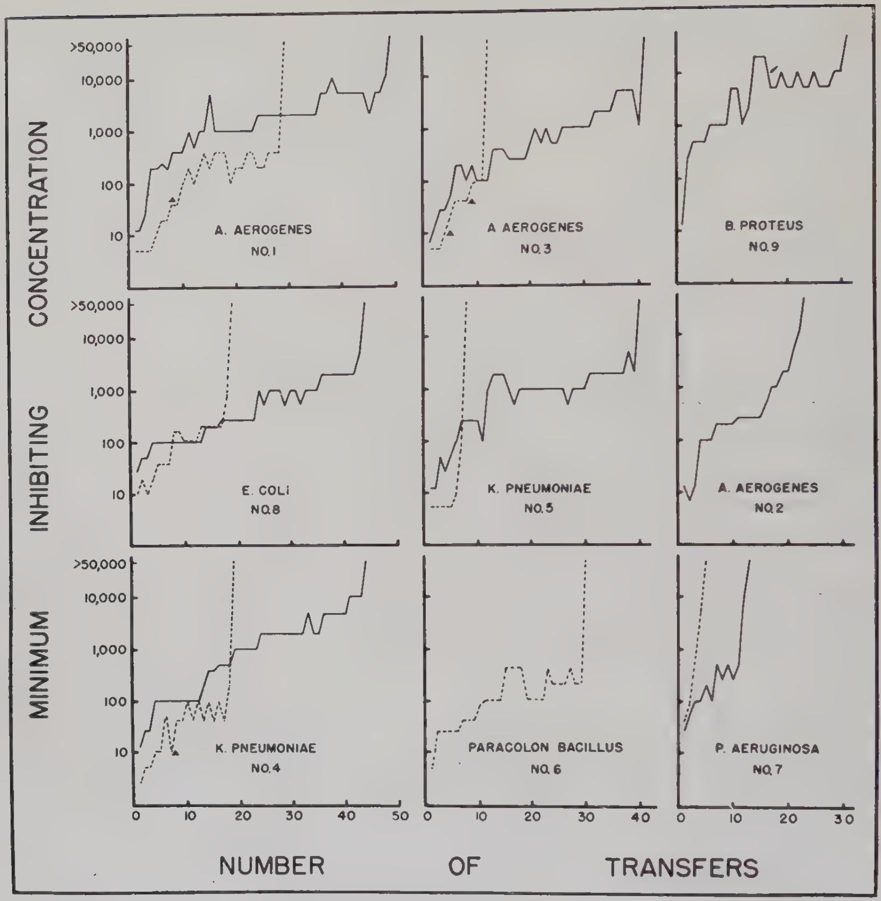


FIG. 1.

Development of resistance in Gram-negative bacilli by transfers through streptomycin-containing broth (solid lines) and agar (broken lines). The solid triangles indicate points at which high resistance developed suddenly in an isolated colony. The minimum inhibiting concentration is given in units (micrograms) per ml.

the others were from cultures of urine in cases of urinary tract infection. Two strains of *Aerobacter aerogenes,* No. 2 and No. 3, obtained from the same patient at different times before treatment, were run through the broth series for comparison and No. 3 was also run on surface plates. Strains 5 and 7 were different organisms from the same patient and both had become resistant during treatment.

Results. Composite results are shown in Table I and Fig. 1. It was possible to raise the resistance of these strains to high values, both in broth and on surface plates. All of the organisms acquired resistance so that they grew well in a concentration of 50,000 units of streptomycin per ml. Varying numbers of transfers were required to accomplish this, ranging from 5 for *Pseudomonas aeruginosa* on surface plates to 49 for a strain of *A. aerogenes* in the broth transfers. Initial sensitivities as indicated by the plate method were lower than those shown in broth by the same strains except in the case of No. 7, a strain of *Ps. aeruginosa,* which developed resistance more rapidly than the other strains.

In the course of the surface plate study a few isolated colonies were noted growing on a concentration of streptomycin higher than the M.I.C. for the rest of the bacterial population of the same culture. Such colonies were picked off, grown in streptomycin-free broth and checked for sensitivity by the broth dilution method. Four of them, shown by solid triangles in Fig. 1, were found to be resistant to over 50,000 units of streptomycin per ml.

There did not seem to be any definite correlation between the rate at which resistance developed *in vitro* and in the patient (Table I). Nor was there any relation between the nature of the organism or its initial sensitivity and the rate of development of resistance *in vitro*. The number of observations, however, are too few to permit any final deductions in this regard.

Organisms in which resistance to streptomycin had been induced as described above were examined and compared, with respect to cultural characteristics and biochemical reactions, with the original sensitive strains and with corresponding resistant strains which were isolated from the patients during the course of treatment. It was previously shown[1] that the original strain and the strain isolated from the patient after therapy were identical morphologically and in their cultural and biochemical characteristics. All organisms which had been passed through streptomycin broth showed some degree of pleomorphism and grew poorly on the usual culture media. The strain of *Escherichia coli* was rough, but was identical in its biochemical reactions with the original sensitive strain and with the corresponding *in vivo* resistant strain. Both of the strains of *K. pneumoniae* grew very poorly in most of the test media used. They failed to ferment saccharose, to produce a Voges-Proskauer reaction or to grow on citrate. Strain No. 5 reacquired the property of fermenting saccharose after 9 passages through broth without loss of resistance to streptomycin but No. 4 failed to do so after 15 such transfers. The remainder of the strains which developed resistance on broth transfer did not appear to differ from the original sensitive strain or from the corresponding *in vivo* resistant strains. Where resistance was acquired on surface plates either by repeated transfer or suddenly in relatively low concentrations of streptomycin, there was less pleomorphism, and, with the exception of the *E. coli* strain, which was rough, could not be distinguished culturally or by biochemical reactions from the corresponding starting or *in vivo* resistant strains. The broth cultures of No. 6 became contaminated during the course of its many transfers and the results are not included.

Discussion. The fact that it was possible to increase the resistance of the organisms studied confirms results described by others.[5-11] Resistance to over 50,000 units of streptomycin per ml represents a figure much higher than has been reported elsewhere and approaches the limit of concentration which can be employed under the conditions of the test. Organisms which are able to grow in this concentration may be regarded as totally resistant. The highest number of transfers required for the induction of such resistance was 49, while the average was 36 for the broth transfer series and 17 for transfer on surface plates. This number, and the time involved, might possibly be reduced were the time per transfer shortened.

The difference in the rates at which resistance developed on surface plates and in broth is of interest. It may be the result of using a larger inoculum in the surface cultures thus offering a larger number of cells of varying resistance (cf. penicillin and staphylococcus[12,13]). This may have some practical implications in connection with the development of resistance by bacteria growing in pockets or walled-off spaces where the concentration of organisms is high and

---

[5] Waksman, S. A., Reilly, H. C., and Schatz, A., *Proc. Nat. Acad. Sc.*, 1945, **31**, 157.

[6] Miller, C. P., and Bohnhoff, M., *J. A. M. A.*, 1946, **130**, 485.

[7] Youmans, G. P., Williston, E. H., Feldman, W. H., and Hinshaw, H. W., *Proc. Staff Meet. Mayo Clin.*, 1946, **21**, 126.

[8] Knop, C. Q., *Proc. Staff Meet. Mayo Clin.*, 1946, **21**, 273.

[9] Alexander, H. E., and Leidy, G., *Science*, 1946, **104**, 101.

[10] Wolinsky, E., and Steenken, W., Jr., PROC. SOC. EXP. BIOL. AND MED., 1946, **62**, 162.

[11] Klein, M., and Kimmelman, L. J., (Abst.) *J. Bact.*, 1946, **51**, 581.

[12] Demerec, N., *Proc. Nat. Acad. Sc.*, 1945, **31**, 16.

[13] Luria, S. E., PROC. SOC. EXP. BIOL. AND MED., 1946, **61**, 46.

that of the antibiotic is relatively low, or in some instances where the exposure of the organism to the antibiotic may resemble that of a surface growth.

The sudden acquisition of extreme resistance by isolated colonies on surface plates suggests that in addition to the gradual increase in resistance shown by organisms exposed to streptomycin, some bacterial cells may suddenly become highly resistant while exposed to comparatively low concentrations. This phenomenon, occurring as it does in material derived from a single picked off colony might offer a partial explanation of the manner in which resistance is acquired *in vivo,* making it unnecessary to postulate the presence of initially resistant strains prior to treatment in those cases where rapid development of extreme resistance is encountered during streptomycin therapy. Failure to observe any sudden increase in resistance in the broth transfer series, however, might have been related to the smaller size of the inoculum used, as judged in terms of numbers of bacteria. The average inoculum in the broth series contained 100,000 to 150,000 organisms while a loopful of surface culture used as the inoculum in the agar plate series contained several billion.

Bacteria developing resistance after prolonged exposure to sublethal concentrations of streptomycin showed impaired growth characteristics. On the other hand, resistant strains from patients under therapy and strains which developed resistance rapidly *in vitro* could not otherwise be distinguished from the strain originally isolated from patients prior to streptomycin therapy.

Conclusions. It was possible to increase the resistance of Gram-negative bacilli to over 50,000 units per ml, in all cases studied, by passage through increasing concentrations of streptomycin in broth and on agar plates.

In most instances resistance increased gradually, the change being more rapid on surface plates than in broth.

In the course of transfers on streptomycin agar, 4 instances of sudden increase in resistance of isolated colonies from relatively low values to over 50,000 units per ml were noted.

The organisms made resistant by exposure to streptomycin in broth showed marked pleomorphism and in some instances suffered changes in their biochemical reactions. When resistance developed as a result of exposure to the agent on surface plates, however, there was less pleomorphism, and no changes in biochemical reactions were noted. Resistant organisms obtained from the patient during or after treatment showed no morphological or cultural differences from the original sensitive strains isolated from the same patient.

15641

Effects of Sodium Fluoride on Hair Growth in the Rat.

EARL O. BUTCHER.

From the Department of Anatomy, College of Dentistry, New York University.

Shour and Smith[1] have shown that intraperitoneal injections of 0.3 cc of 2.5% sodium fluoride at 48-hour intervals induced hypocalcified stripes in the enamel and dentin of rats. When the injections were given at intervals of 24 hours or the dosage was increased, cumulative effects were produced which severely disturbed the formation of enamel and dentin. The effects produced by adding NaF to the diet were essentially similar to the effects produced by injections. Since the cytoplasm of the ameloblasts showed an abnormal character and disturbance of its globules, Shour and Smith suggested that fluorine exerted a direct local action on the enamel-forming cells.

---

[1] Shour, I., and Smith, M. C., *University of Arizona Agr. Exp. Sta. Tech. Bull.*, 1934, **52**, 69.

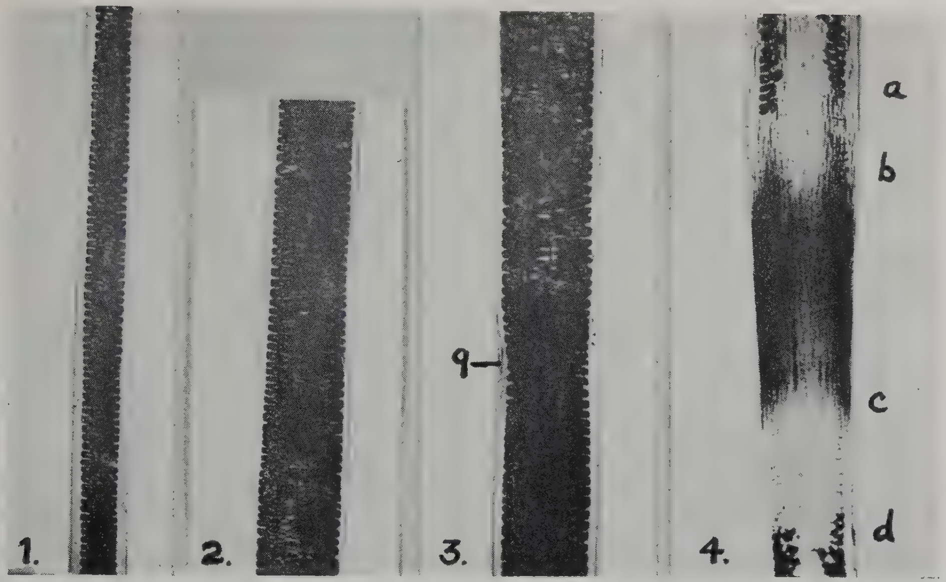


FIG. 1. Coarse hair from a rat whose diet contained 0.1% NaF.

FIG. 2. Coarse hair from a control litter mate. Photographs 1 and 2 were taken the same distance from the tips of the hairs.

FIG. 3. This photograph shows an interval of a hair which was affected by the intraperitoneal injection of 0.15 cc of 2.5% NaF. The medulla consists of fewer cells and granules (g) have formed in the cortex.

FIG. 4. The affected area resulting from the intraperitoneal injection of 0.2 cc of 2.5% NaF extends from a to d. The medulla is transparent due to little air between the cells and the granules in the cortex (b to c) mask the central medullary cells.

All photographs have the same magnification of 110 ×.

The present study has been made to see if another ectodermal derivative, the hair, was affected in any way by sodium fluoride. If NaF administration induced any effects on the hair, it also seemed worthwhile to learn if these effects differed from those resulting from inanition.

Hair was removed from the backs of 15 23-day-old rats. Five of these served as controls and received Purina Dog Chow while 10 were fed finely ground chow containing 0.1% NaF. Similar young rats receiving a 0.2% concentration in their diet failed to grow and survive well. The average gain in weight of those animals receiving 0.1% NaF in their diet was 10 g between the 23rd and 40th days of life, and the average gain of the 5-litter-mate controls over the same period was 45 g. Hair grew on the controls on the 35th or 36th day of life as described in a previous paper[2] while hair which grew slowly and evenly was retarded on the average of 10 days in the NaF-fed animals. A measurement of 50 of the coarser hairs of the treated animals showed that they had only 60% of the length and 60% of the diameter of similar hairs of the controls. Photographs of hairs from the NaF-fed rat (Fig. 1) and a control animal (Fig. 2) were taken the same distance from the tips of the hairs and are presented for comparison. The medulla of the hair of the NaF-fed animal consists of fewer cells while the cortex seems little affected. Histological sections fixed in Bouin's and stained with hematoxylin and eosin failed to show cellular disturbances in the hair bulb which would account for this small size. The teeth of the NaF-fed animals gradually became pigment-free and chalky white.

In order to compare the effects of inanition with the effects of NaF in the feed, 6 23-day-old rats were fed daily 5 g of finely ground chow as in previous experiments.[3]

---

[2] Butcher, E. O., *Anat. Rec.*, 1934, **61**, 5.

[3] Butcher, E. O., *J. Nutrition*, 1939, **17**, 151.

Such rats gained 8 g in 16 days as compared to the 10 g average gain made by the NaF-fed animals in a similar period. Hair growth was retarded about 10 days in the underfed animals and appeared in small areas over the dorsum in comparison to the even growth of 3 well-fed litter mates. A measurement of the diameters of 50 hairs showed that in many instances the hairs were as large as those of the controls and seldom were 10% smaller. All the hair follicles in the NaF-fed animals appeared to be affected while only localized areas of follicles in the underfed animals seemed to receive enough nutrition to produce hair. The teeth of the underfed animals erupted nearly as fast as those in the controls, keeping their typical translucent appearance. The eruption rate for the lower incisors in underfed and well-fed rats averaged about 0.6 mm daily. This rate varies from that reported by Addison and Appleton[4] (0.4 mm daily) and Smith[5] (0.45 mm daily).

Shour and Smith[1] injected 0.3 cc of a 2.5% solution intraperitoneally in 90- to 270-day-old rats. In the present experiments 35-day-old rats would usually tolerate 0.3 cc of a 1.2% solution intraperitoneally. It was found best to introduce 0.2 cc of this 1.2% solution and then to administer 2 more injections of 0.05 cc at half-hour intervals. Some animals did tolerate 0.2 cc of a 2.5% solution. Such injections were made in 38- to 42-day-old rats or when the second growth of hair was ⅛ to ¼ inches long externally (the first growth was removed at the age of 23 days with sodium sulphide). Forty-eight hours after the injection, hairs were plucked, dried, and mounted in clarite.

The administration of the NaF affected the hair while it was forming and when the affected area emerged from the follicle, the medulla was narrowed for a short interval (Fig. 3). The medulla of this interval consisted of fewer cells which is similar to the effects resulting from feeding the NaF. Injections every 48 hours caused successive narrowings in the hair. In many instances the effects were greater and the affected area was much longer as shown in Fig. 4 where it extends almost the entire length of the microphotograph. Fewer cells constituted the medulla and the cells often failed to shrink. Accordingly, there was less space for air between the cells. When hairs were mounted in clarite, the medulla in the normal region appeared black due to the large amount of air between the cells while the interval affected by the NaF appeared transparent.

In addition to the effects on the medulla there was in most cases formation of granules in the cortex (Fig. 3). In Fig. 4 the medulla is transparent from a to d due to the small intercellular spaces. The granules in the cortex are so numerous and black that they mask the central medullary cells from b to c.

The injections thus caused greater effects than the feeding. Nembutal anesthesia for many hours produced no effects on the hair similar to those caused by the injection of NaF.

Summary. Inclusion of 0.1% NaF in the diet of rats resulted in the retardation of the growth of hair and in smaller hairs. Underfeeding, so that the gain in weight was similar to that when NaF was included in the diet, did not produce a similar reduction in the size of the hair.

Intraperitoneal injections of NaF affected the growth of the hair and caused a narrowing of the hair for a short interval. The medulla of this interval consisted of fewer cells. The medullary cells also often failed to shrink, resulting in little intercellular space between them. In addition, the intraperitoneal injections caused the formation of granules in the cortex. Since NaF is commonly accepted as an inhibitor of acid phosphatase,[6] it may be that NaF affects hair growth through inhibition of this enzyme.

---

[4] Addison, W. H. F., and Appleton, J. L., *J. Morph.*, 1915, **26**, 43.

[5] Smith, M. C., *J. Dent. Res.*, 1934, **14**, 139.

[6] Moog, Florence, *Biol. Bull.*, 1944, **86**, 51.

15642 P

Electron Micrographs of the Elementary Bodies of Fowl Pox and Canary Pox.

VINCENT GROUPE, JOHN OSKAY, AND GEOFFREY RAKE.
From the Squibb Institute for Medical Research, New Brunswick, N.J.

The virus of vaccinia was the first of the pox group to be studied with the electron microscope. Pictorial data presented by Green, Anderson and Smadel[1] showed that the elementary bodies are roughly rectangular in shape, possess a limiting membrane and exhibit internal differentiation as shown by the presence of rounded areas of greater density than the remainder of the particle. These findings were confirmed by Sharp, Taylor, Hook and Beard[2] who, in addition, utilized the shadow-casting technic of Williams and Wyckoff[3] and presented evidence that the elementary bodies are essentially short cylinders. Inasmuch as the above studies have shown the elementary bodies of vaccinia to have definite and distinctive morphological characteristics, it was of interest to compare the morphology of the elementary bodies of fowl pox and canary pox with those of vaccinia.

Materials and Methods. Egg-adapted strains of fowl pox,* canary pox* and vaccinia† were studied in each case. Suspen-

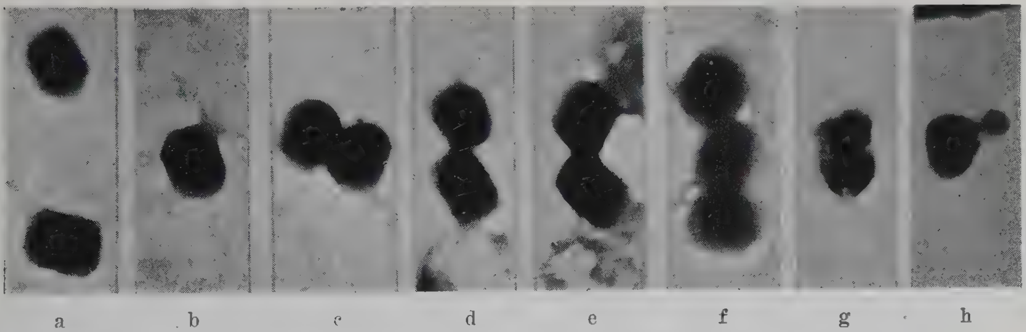

a b c d e f g h

FIG. 1.
Elementary bodies of fowl pox. Magnification 29,200 ×.

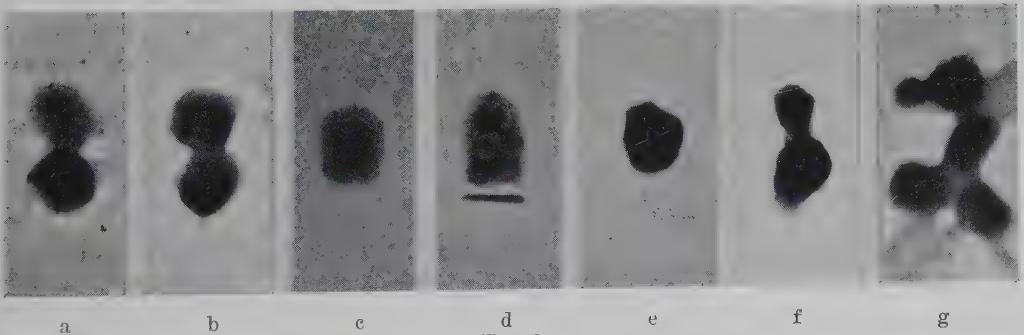

a b c d e f g

FIG. 2.
Elementary bodies of canary pox. Magnification 29,200 ×.

---

[1] Green, R. H., Anderson, T. F., and Smadel, J. E., *J. Exp. Med.*, 1942, **75**, 651.

[2] Sharp, D. G., Taylor, A. R., Hook, A. E., and Beard, J. W., PROC. SOC. EXP. BIOL. AND MED., 1946, **61**, 259.

[3] Williams, R. C., and Wyckoff, R. W. G., PROC. SOC. EXP. BIOL. AND MED., 1945, **58**, 265.

* Strains obtained through the courtesy of Dr. F. R. Beaudette of the New Jersey Experiment Station, New Brunswick, N.J.

† Strain obtained through the courtesy of Dr. J. B. Nelson of the Rockefeller Institute for Medical Research, Princeton, N.J.

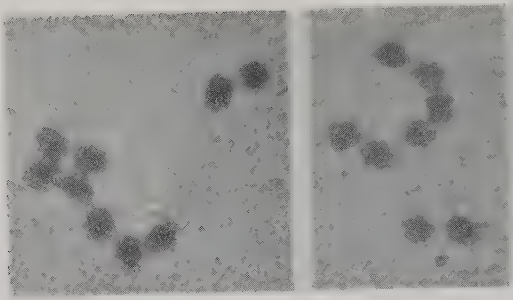


FIG. 3.
Canary Pox. Magnification 20,400 ×.

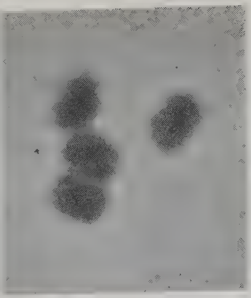


FIG. 4.
Vaccinia. Magnification 29,200 ×.

sions for examination in the electron microscope were prepared from heavily infected chorioallantoic membranes inoculated by Burnet's technic.[4] The infected membranes were ground with sand and 10% suspensions in saline prepared. Partial purification of the elementary bodies was obtained by 2 or 3 cycles of differential centrifugation at speeds of 1,000 r.p.m. and 15,000 r.p.m. for 15 minutes and 45 minutes respectively. A small drop of the virus suspension was then placed on the collodion film support and the excess fluid removed with a fine pipette. After drying the film was washed with 3 drops of distilled water and again allowed to dry. The dried films were then examined in an RCA electron microscope (type EMU).

Results. It will be seen from the pictorial data presented in Fig. 1 and 2 that the elementary bodies of fowl pox closely resemble those of canary pox. Furthermore, although the elementary bodies of vaccinia (presented for comparison in Fig. 4) are somewhat smaller and narrower than those of fowl pox and canary pox, morphological characteristics observed in the elementary bodies of vaccinia[1,2] are also evident in those of fowl pox and canary pox. The particles of these avian poxes are approximately rectangular in shape (Fig. 1a-c and 2a-d) and like vaccinia the elementary bodies of these avian poxes occur singly, in pairs and in short chains (Fig. 3). However, the arrangement of the particles in pairs or chains is subject to considerable variation. Particles are most frequently attached to one another by their corners (Fig. 1d, e and 2a, b, g) but are occasionally attached side to side (Fig. 1c) or end to end (Fig. 3). The characteristically flattened corner frequently observed on many particles (Fig. 1b and 2c, e) and visible in the micrographs of vaccinia presented by other investigators,[1,2] although not specifically commented on by them, probably results from the separation of 2 particles joined by their corners. Curious bud-like structures like those shown in Fig. 1h and 2 f, g, which usually also appear at the corners of the particle, are observed sufficiently frequently to lead to the belief that they are of significance. Studies on the nature of these structures are in progress.

Summary. The elementary bodies of fowl pox closely resemble those of canary pox and have many morphological characteristics in common with the elementary bodies of vaccinia.

---

[4] Burnet, F. M., *Sp. Rep. Ser. Med. Res. Counc., London,* No. 220, 1936.

15643

Effects of Massive Doses of P-Aminobenzoic Acid and Inositol on Reproduction in the Rat.

BENJAMIN H. ERSHOFF.
From the Emory W. Thurston Laboratories, Los Angeles, Calif.

A few years ago Martin[1] found that although rats grew adequately on purified rations containing 6 B complex factors (thiamine, riboflavin, pyridoxine, choline, nicotinic acid and calcium pantothenate), addition of inositol resulted in retardation of growth and a number of early deaths. Similar adverse effects were noted following the administration of *p*-aminobenzoic acid but not when *p*-aminobenzoic acid and inositol were fed concurrently. Subsequent studies with rats have not confirmed these results,[2] although Sure[3] observed a toxic effect of inositol on lactation and Ershoff and McWilliams[4] noted reduced fertility when inositol was fed along with purified rations containing both *p*-aminobenzoic acid and sulfaguanidine. In the present experiment rats were raised to maturity on purified rations containing massive doses of *p*-aminobenzoic acid or inositol and the adequacy of such diets for reproduction was determined.

Procedure and Results. Forty-two female rats of the Long-Evans strain were selected at 23 days of age and an average weight of 41.8 g. They were kept in metal cages with raised screen bottoms to prevent access to feces and were fed *ad libitum* the 7 diets listed in Table I (6 animals per group). They were weighed weekly, and vaginal smears were taken daily from the 48th day. After the 60th day the animals were bred to males of proven fertility.

Growth. No significant difference in rate of growth or gross appearance was observed except perhaps in the rats fed diet C. After 60 days the animals on this diet averaged 173.8 g in weight; average weights for those on the other diets ranged from 184.2 g on diet G to 204.8 g on diet F.

Reproduction. No significant difference in reproductive performance was observed.

TABLE I.
Composition of Experimental Diets.

Dietary component	A	B	C	D	E	F	G
Casein*	30.0	30.0	30.0	30.0	30.0	30.0	30.0
Salt mixture†	4.5	4.5	4.5	4.5	4.5	4.5	4.5
Sucrose	65.5	64.5	63.5	64.5	63.5	65.5	55.5
p-aminobenzoic acid		1.0	2.0		1.0		
Inositol				1.0	1.0		
Folic acid						0.0005	
Yeast‡							10.0

To each kg of diets A-F, were added 80 mg thiamine hydrochloride, 20 mg riboflavin, 20 mg pyridoxine hydrochloride, 100 mg calcium pantothenate, 100 mg nicotinic acid, 5 mg 2-methylnaphthaquinone and 1.2 g choline chloride. To each kg of diet G were added 5 mg 2-methylnaphthaquinone and 1.2 g choline chloride. In addition each rat on diets A-G received the following supplement 3 times weekly: 500 mg cottonseed oil (Wesson), 1.0 mg alpha-tocopherol, and a vitamin A-D concentrate containing 50 U.S.P. units of vitamin A and 50 U.S.P. units of vitamin D.‖

* Vitamin Test Casein, General Biochemicals, Inc., Chagrin Falls, Ohio.

† Sure's Salt Mixture No. 1.[5]

‡ Brewers' type yeast No. 200, Anheuser-Busch, Inc., St. Louis, Mo.

‖ Nopco Fish Oil Concentrate, assaying 800,000 U.S.P. units of vitamin A and 80,000 U.S.P. units of vitamin D per gram.

---

[1] Martin, Gustav J., *Am. J. Physiol.*, 1942, **136**, 124.

[2] Ershoff, B. H., PROC. SOC. EXP. BIOL. AND MED., 1944, **56**, 190.

[3] Sure, Barnett, *J. Nutrition*, 1943, **26**, 275.

[4] Ershoff, B. H., and McWilliams, H. B., PROC. SOC. EXP. BIOL. AND MED., 1943, **54**, 227.

[5] Sure, Barnett, *J. Nutrition*, 1941, **22**, 499.

All the rats cast litters, although in 3 instances the initial breeding proved unsuccessful. Number of young per litter varied from 2 to 13, with averages between 7.2 and 8.4 on the several diets. An average of 0.8 to 1.2 young per litter were born dead (omitting one litter on diet C which consisted of 9, all born dead). In all groups the young averaged 5 to 5½ g at birth and appeared grossly to be normal in all respects.

Lactation. At birth litters were reduced to 6 each. The young were nursed and weaned on all diets except E and F. On each of diets B, D and G, 3 mothers successfully nursed their young, which were weaned at 21 days of age at average weights of 36 to 41 g. Two litters on diet A were nursed, but only 5 of the 12 young survived until weaning (average weight at weaning, 22.8 g). The one litter weaned on diet C consisted of 3 survivors, average weight 24.6 g.

The findings indicate that the massive doses of inositol or *p*-aminobenzoic acid exerted no deleterious effects on growth or reproduction and that lactation may occur on diets containing 1% *p*-aminobenzoic acid or inositol. The cause of the poor lactation is unknown, but deficiency of folic acid was probably not a factor. To judge from the work of Maynard and Rasmussen,[6] failure of lactation may have been due in part to insufficiency of dietary fat.

Summary. No adverse effects on growth or reproduction were noted in female rats fed purified rations containing 1% *p*-aminobenzoic acid or inositol. Lactation was inadequate both in control and experimental rations, although some young were weaned on each of the above diets.

---

[6] Maynard, L. A., and Rasmussen, E., *J. Nutrition*, 1942, **23**, 385.

15644 P

Relief of Cardiac Pain by Local Block of Somatic Trigger Areas.

JANET TRAVELL AND SEYMOUR H. RINZLER.

From the Cardiac Service, Beth Israel Hospital, and the Department of Pharmacology, Cornell University Medical College, New York City.

Weiss and Davis[1] noted that referred pain from viscera could be abolished by procaine infiltration of tender areas in the somatic reference zone. Their observations became buried, however, in the controversy which developed over the mechanism of visceral pain reference and the role of afferent somatic impulses in this phenomenon. As a result, the therapeutic implications of their results have received little recognition. In agreement with their findings, our data indicate that under suitable conditions, cardiac pain may be abolished by local block of the noxious impulses which arise in trigger areas in the reference zone.

Procaine Infiltration. We became interested in this problem during studies on extracardiac sources of pain in the chest and arm, which in some respects simulates coronary artery pain.[2] By the technic of needling tender spots in the muscles in patients with various myalgic syndromes, we discovered the existence of a group of trigger areas, located chiefly along the sternal borders, mechanical stimulation of which uniformly induced a reference of pain perceived beneath the sternum and deep inside the chest; if located on the left side the referred pain was often described as "felt directly in the heart." Other trigger areas in the chest and shoulder regions induced a radiation of pain to the entire pectoral area, the interscapular region, neck and arm.

At that time we believed[2] that since local injection therapy afforded relief in such patients without organic heart disease, this procedure might serve as a therapeutic test to exclude a visceral etiology of pain. However, when we subsequently applied this technic for diagnostic purposes, we concluded that local infiltration of somatic trigger areas probably relieved the pain of both myalgias and effort angina. In 2 patients the angina of effort disappeared during the course of a series of such treatments. In one patient in an almost continuous anginal status, the nocturnal anginal attacks, which required repeated doses of glyceryl trinitrate, almost entirely disappeared after 2 treatments. The daytime attacks in this and in 2 other patients were apparently benefitted for only short periods of time after each treatment.

---

[1] Weiss, S., and Davis, D., *Am. J. Med. Sci.*, 1928, **176**, 517.

[2] Travell, J., Rinzler, S., and Herman, M., *J. A. M. A.*, 1942, **120**, 417.

Because of the difficulties in evaluating results of therapy in chronic cardiac pain, we selected 4 patients with acute pain due to a recent myocardial infarct for a therapeutic trial. Three episodes of acute myocardial infarction (2 attacks in one patient 2 years apart) were treated by local procaine infiltration of the somatic trigger areas in the anterior chest wall, in about 7 hours, 13 hours and 21 days, respectively, after the onset of pain which was not completely relieved by morphine. In each instance this procedure afforded immediate, complete and permanent relief of chest pain and its associated radiation.

Ethyl Chloride Spray. The excellent results obtained by ethyl chloride spray in acute myalgias[3] and in sprains[4–7] indicate that this procedure, like procaine infiltration, blocks noxious impulses from somatic trigger areas following injury, provided the trigger areas are not deeply located beneath the body surface. We inferred, therefore, that ethyl chloride spray of trigger areas in the pectoral muscles, because fairly superficially located, might also abolish the pain of acute myocardial infarction. We found that when the major precordial trigger area was sprayed with ethyl chloride, the constant pain of a fresh myocardial infarct (23 hours old), unrelieved by demerol and papaverine, was in one case permanently abolished within 20 seconds after the start of spraying. It seems likely that relief afforded by ethyl chloride spray is at least in part due to refrigeration anesthesia of the myofascial structures, for in another patient our data suggest that if the trigger area is situated as much as one inch below the surface of the skin, it may be necessary to substitute injection with procaine for ethyl chloride spray.

Mechanisms. A parallelism is apparent between mechanisms of pain following trauma to the somatic structures, as in sprains, and mechanisms involved in pain due to acute myocardial infarction, since pain following both types of injury can be relieved by local block of somatic trigger areas. The most reasonable explanation is that the initial insult, whether to visceral or somatic structures, sets in motion a chain of events perpetuated by a vicious cycle of nerve impulses which have no further dependence on afferent impulses from the heart and which are probably transmitted to and from the soma by virtue of sustained facilitation of the noxious impulses by the closed self-reexciting chains of internuncial neurons in the central nervous system.[8–10]

Certain similarities in the quality and behavior of referred somatic and referred visceral pain,[11–13] together with the results of local anesthesia of somatic structures, indicate that the physiologic mechanisms by which the body expresses pain are probably the same for visceral and somatic disease, and as postulated by Weiss and Davis,[1] in both instances involve the soma as an integral part of the reference of pain. In this connection, it is important to recognize that disappearance of pain after blocking the somatic component does not exclude the diagnosis of visceral disease as the initiating cause of pain.[14] There is even some evidence that reflex coronary vasodilatation may follow the termination of the vicious cycle by precordial local anesthesia and that the visceral lesion is thus subject to influences from somatic trigger mechanisms.[15]

---

[3] Kraus, H., *J. A. M. A.*, 1941, **120**, 417.

[4] Cozen, L. N., and Hollombe, B. S., *Surgery*, 1940, **8**, 648.

[5] McIntosh, C. A., and Petrie, J. G., *Lancet*, 1942, **2**, 279.

[6] Henry, A. K., *Lancet*, 1942, **2**, 280.

[7] Bingham, R., *Mil. Surgeon*, 1945, **96**, 170.

[8] Lorente de Nó, R., *J. Neurophysiol.*, 1938, **1**, 207.

[9] Evans, J. A., *Surg., Gyn., and Obst.*, 1946, **82**, 36.

[10] Livingston, W. K., *Pain Mechanisms*, The Macmillan Company, 1943, p. 224, Chap. XV.

[11] Lewis, T., and Kellgren, J. H., *Clin. Sci.*, 1939, **4**, 47.

[12] Kellgren, J. H., *Clin. Sci.*, 1939, **4**, 35.

[13] Travell, J., and Bigelow, N. H., *Fed. Am. Soc. Exp. Biol. (Proc.)*, 1946, **5**, 106.

Conclusion. Data secured in 9 patients with effort angina and acute myocardial infarction indicate that local block of somatic trigger areas by procaine infiltration or ethyl chloride spray under suitable conditions may afford complete and prolonged relief of pain. Because of their simplicity and effectiveness, these procedures deserve extensive clinical trial.

---

[14] Gorrell, R. L., *Am. J. Surg.*, 1944, **63**, 102.

[15] Lindgren, I., *Nordisk Medicin*, 1946, **29**, 523; (Abs.) *J. A. M. A.*, 1946, **131**, 1536.

15645 P

Toxic Effects in Scorbutic Guinea Pigs Produced by Large Doses of Thiamin.*

DORIS M. HIGHET AND EDWARD S. WEST.

From the Department of Biochemistry, University of Oregon Medical School, Portland, Oregon.

During the study of the effects of administration of thiamin to scorbutic and non-scorbutic animals, it was observed that young scorbutic pigs fed large amounts of thiamin sometimes develop irreversible symptoms after the resumption of a normal diet. The strength of the jaw muscles decreases and the animals are unable to chew their food properly, although they appear hungry and persistently try to eat. There is also decreased control of jaw muscles and the animals often spend long periods with jaws constantly opening and closing in a rapid chewing motion. This condition sometimes becomes permanent until death. The lack of muscle control is also often manifested by a shivering movement of the face accompanied by chattering of the teeth. In some cases the mouth is distorted and the muscles involved are in spastic contraction, causing the animals to claw the face in attempts to relieve the spasm. These muscle dyscrasias are usually accompanied by increased salivation or loss of saliva which may be due to difficulty in swallowing.

The symptoms, once established, have been found irreversible and the animals inevitably die. Attempts to avert death by forced feeding, administration of large amounts of synthetic ascorbic acid or by offering green food have failed to keep the animals alive longer than one month.

Death is not primarily due to starvation, for if the food offered is well macerated these animals consume amounts normal for their size. At death the animals are extremely emaciated. (Pigs weighing 400 g initially, decreased to 270-300 g). At autopsy macroscopic hemorrhagic areas are found to vary inversely with the length of time the animals have lived after the onset of symptoms.

Several methods of producing the thiamin effect have been tried. The procedure giving the highest incidence was as follows:

Animals were fed a diet of Olympic rabbit pellets and water, until weight gain ceased and loss of appetite, joint stiffness and tenderness were observed. The time required for these signs to appear was about 15 days. At that time the daily diet was supplemented by oral administration of 25 mg of thiamin hydrochloride in aqueous solution. When the animals became acutely ill with scurvy, usually after 10 days, the thiamin hydrochloride was withdrawn and green food was offered freely. The animals were allowed this diet until definite signs of improvement were seen. At that time the green food was removed, administration of thiamin hydrochloride was resumed and the animals were again brought to the stage of acute scurvy. Repeated treatment in this sequence was employed.

---

* Taken from a thesis submitted by Doris M. Highet in partial fulfillment of the requirements for the degree of Doctor of Philosophy. Aided by a grant from the General Research Council of the Oregon State System of Higher Education.

The thiamin effect has been produced in 12 pigs by this procedure, or in roughly one-third of the animals subjected to the treatment. The effect has also been observed in 6 pigs made scorbutic and given thiamin according to variations in the above method.

No explanation for the symptoms or cause of death can be given at this time. Death was apparently not due to starvation, for when food was offered in macerated form enough was ingested to maintain life. Death did not appear to be due to scurvy *per se,* for animals maintained on macerated green food after the development of the symptoms and which had lived for a time after being placed on this diet did not show the usual signs of scurvy at autopsy. Death appears to have been due to some irreversible effect caused by the superimposition of large amounts of thiamin during scurvy. We have not succeeded in producing apparent toxic effects in normal guinea pigs by giving large doses of thiamin.

Pigs have been most susceptible to the thiamin effect during the late winter months.

Histological study of brain sections and peripheral nerves is in progress in an attempt to discover a possible neurological cause of the symptoms and death.

Apparently this effect of large doses of thiamin upon scorbutic animals has not been observed previously.

15646

Hypoglycemic Action of Sulfanilamido-cyclopropylthiazole in Rabbits, and Its Reversal by Alloxan.

K. K. CHEN, ROBERT C. ANDERSON, AND NILA MAZE.

From the Lilly Research Laboratories, Eli Lilly and Company, Indianapolis.

Janbon and his co-workers[1] showed that in man sulfanilamido-isopropylthiadiazole caused a fall of blood sugar, to such an extent that hypoglycemic shock occurred. Loubatières,[2] working with the same compound in dogs, reported that hypoglycemia appeared when the product was given by mouth or by subcutaneous injection. In depancreatized dogs, however, no drop of blood sugar was observed in spite of increased dosage. The author thus concluded that this thiadiazole stimulated insulin secretion, and that this action disappeared if the islets of Langerhans were absent. Bovet and Dubost,[3] and Loubatières[4] studied several analogues of sulfanilamido-isopropylthiadiazole, and found that the butyl, isobutyl, and amyl derivatives were more potent than the isopropyl.

Doctors L. P. Kyrides and F. B. Zienty, of Monsanto Chemical Co., St. Louis, Mo., generously supplied us with a compound closely related to the French product, and suggested an investigation of its effect on blood sugar

---

[1] Janbon, M., Lazerges, P., and Métropolitanski, J. H., *Montpelier Méd.*, 1942, **21-22**, 489.

[2] Loubatières, A., *C. R. Soc. de biol.*, 1944, **138**, 766.

[3] Bovet, D., and Dubost, P., *C. R. Soc. de biol.*, 1944, **138**, 764.

[4] Loubatières, A., *C. R. Soc. de biol.*, 1944, **138**, 830.

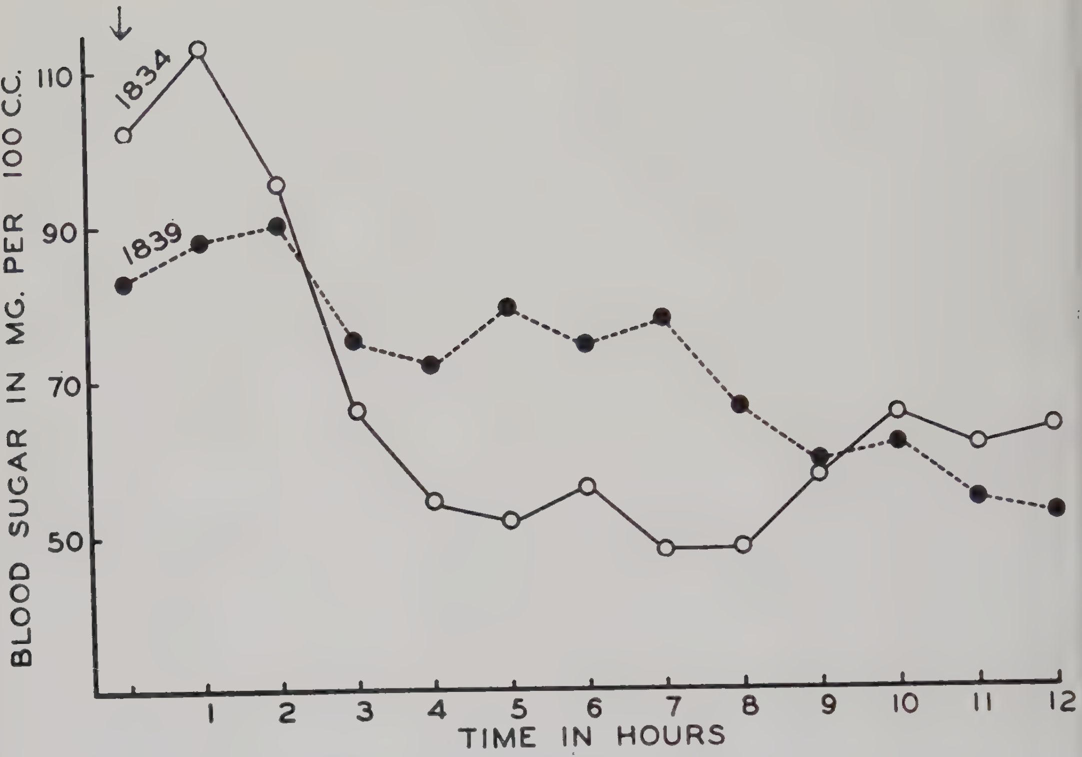
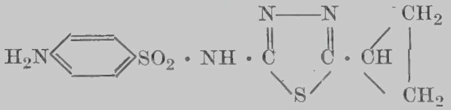


FIG. 1.

Hypoglycemic Action in Normal Rabbits.

The blood sugar readings of 2 rabbits are plotted in the chart. Both rabbits were male. At arrow, 2-sulfanilamido-5-cyclopropyl-1,3,4-thiadiazole suspended in water was given by stomach tube—the dose being 1 g per kg. No convulsions took place at the height of hypoglycemia.

in addition to its other properties. Its chemical name is 2-sulfanilamido-5-cyclopropyl-1,3,4-thiadiazole, and has the following formula:



The material melts at 221-222°C, and is very slightly soluble in water.

The present compound, like many sulfonamides, has a chemotherapeutic action against bacteria. According to the unpublished results of Dr. H. M. Powell of our own laboratories, it inhibits the growth of streptococcus and pneumococcus, type I, *in vitro,* and is capable of saving lives of mice infected with pneumococcus, type I, dysentery bacillus, and hemolytic streptococcus.

When given by mouth to rabbits in the dose of 296 mg per kg, it was appreciably absorbed but not to the same extent as sulfadiazine. The peak of blood concentration of the free form with that dose varied from 3 to 6.5 mg per 100 cc in 6 hours. No conjugation could be demonstrated in the blood stream. The toxicity of 2-sulfanilamido-5-cyclopropyl-1,3,4-thiadiazole is low—failing to kill mice in a single dose of 25 g per kg given orally.

Six groups of 5 rats each were fed diets containing 0.25, 0.5, 1.0, 1.5, 2.0, and 2.5% of this substance, respectively, for 22 days. All animals survived. Upon sacrifice, no renal lesions were discovered in spite of the high blood concentration, which varied from 20 to 23.5 mg per 100 cc. Lack of con-

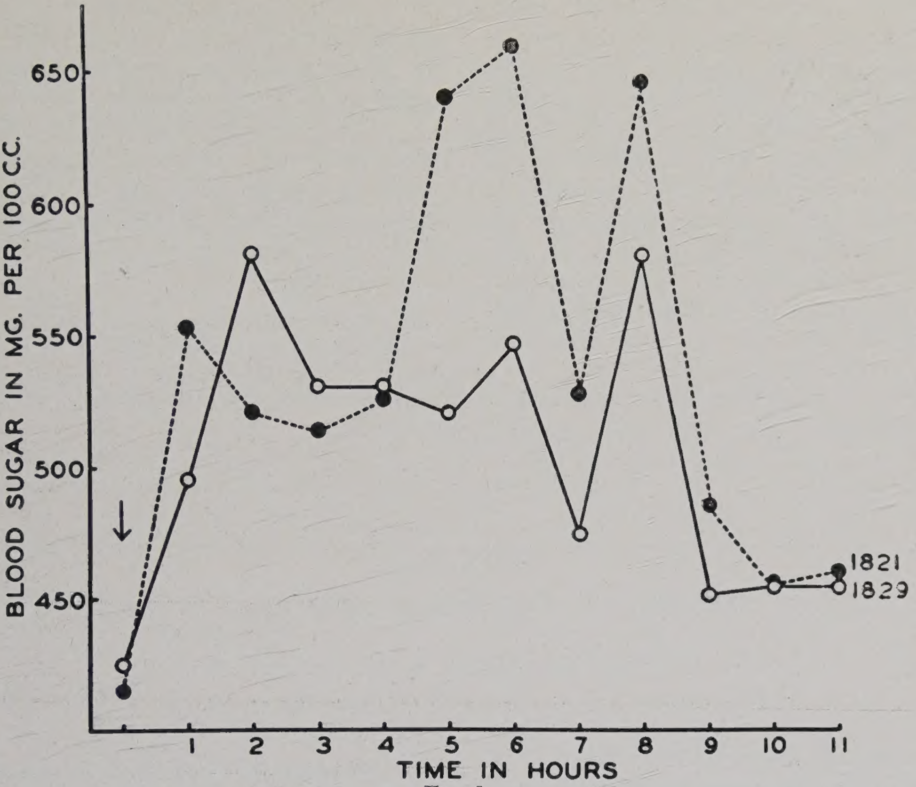


FIG. 2.

Hyperglycemic Action in Diabetic Rabbits.

Diabetes of rabbits was induced by intravenous injection of alloxan. Rabbit No. 1829 received a dose of 175 mg per kg; and the other, No. 1821, 200 mg per kg. Glucose in the amount of 2 g per kg was administered following alloxan at 2-hour intervals until each rabbit recovered from the crisis. At arrow, 2-sulfanilamido-5-cyclopropyl-1,3,4-thiadiazole was given *per os* to rabbit No. 1829 in the dose of 1 g per kg, and to rabbit No. 1821 in the dose of 2 g per kg. The rise of blood sugar in each case is clearly shown by the curves.

jugation probably accounts for the absence of kidney damage. The only change uniformly observed was hypertrophy of the thyroid gland, which also occurs with other sulfonamides.[5]

The most unique feature of the compound is its hypoglycemic action following oral administration. Our experiments were carried out in rabbits. Fig. 1 shows the blood sugar curves of 2 starved animals, the method of Hagedorn and Jensen[6] being employed for the sugar determination. It should be noted that a dose of 1 g per kg reduced the blood sugar to as low as 48 mg per 100 cc in one, and 52 mg per 100 cc in the other, with a slight initial rise of blood sugar. Four additional rabbits received a smaller dose, 0.6 g per kg each, and all exhibited a fall of blood sugar without primary hyperglycemia.

A second group of rabbits was made chronically diabetic with alloxan according to Duffy's technic.[7] In our hands, about one-half of the animals died before diabetes was established. Six experiments were performed on 5 diabetic rabbits with oral administration of 2-sulfanilamido-5-cyclopropyl-1,3,4-thiadiazole. The dose varied from 1 to 2 g per kg. The results of 2 of them are charted in Fig. 2. It is obvious that an opposite effect occurred, namely, a rise of blood sugar. The figures of the latter were so high that aliquot portions of each blood sample had to be used. The data from the remaining 4 experiments are closely similar to those shown in Fig. 2. Four of the 5 diabetic rabbits were sacrificed and submitted to necropsy. Necrosis and disappearance of pancreatic island tissues were evident. It thus becomes clear that our product, like the French, stimulates insulin secretion in rabbits with normal pancreas. The explanation for the occurrence of hyperglycemia in diabetic rabbits following the same compound requires further investigation.

---

[5] Mackenzie, C. G., and Mackenzie, J. B., *Endocrinology*, 1943, **32**, 185.

[6] Hagedorn, H. C., and Jensen, B. N., *Biochem. Z.*, 1923, **135**, 46.

[7] Duffy, E., *J. Path. and Bact.*, 1945, **57**, 199.

Summary. 1. 2-Sulfanilamido-5-cyclopropyl-1,3,4-thiadiazole, like other sulfonamides, has a chemotherapeutic action against certain pathogenic bacteria. It has a low toxicity. There is no evidence of conjugation as judged by blood examination.

2. When given to normal rabbits, the substance uniformly causes hypoglycemia—with or without a slight initial rise of blood sugar. On the other hand, in diabetic rabbits induced by alloxan, it produces a definite rise of blood sugar.

The authors are indebted to Dr. Paul N. Harris for his post-mortem examination of our diabetic rabbits, and to Miss Marian H. Ellaby and Mr. Harold M. Worth for their assistance in making the charts.

15647 P

Glycolysis in *Trypanosoma equiperdum.*

Graham Chen and E. M. K. Geiling

From the Department of Pharmacology, University of Chicago.

For some time there has been doubt as to whether a phosphorylative mechanism is involved in the breakdown of glucose in *Trypanosoma equiperdum.*[1-3] The purpose of this communication is to report experiments concerned with the metabolic pathway involved in the degradation of glucose by a blood-free preparation of lysed *Trypanosoma equiperdum.** Such a preparation was found to transform glucose into fructose 1, 6-diphosphate by phosphorylation, and to catalyze the splitting of fructose 1, 6-diphosphate and the coupled oxidation-reduction of 3-phosphoglyceraldehyde into phosphoglyceric

TABLE I.

The Transformation of Glucose into Fructose and Triose Phosphates by Lysed *T. equiperdum.*

The system contained 0.015 M $NaHCO_3$, 0.003 M $MgSO_4$, 0.015 M NaF, 0.015 M KCl, 0.012 M glucose, 0.003 M adenosine triphosphate (tipped in from side arm after equilibration), and 0.4 cc of a lysed preparation (equivalent to about 3 × 10^8 parasites) in a total volume of 2.0 cc in Warburg flasks; gas phase 5% CO_2-95% N_2; temperature 38°C; time of experiment, 1 hr; 0.2 cc of 100% trichloracetic acid solution added to terminate the reaction.

Glucose utilized,	CO_2 evolved,	Fructose formed,	ATP split (7 min. acid P.),	*20 min. alk. P. formed,	†Increase of 2 hour acid P.,
μg	μl	μg	μg	μg	μg
1176	15	779	107	21	50

* Triose phosphates.

† Glucose-6-phosphate and fructose 1-6-phosphate.

---

[1] Reiner, L., Smythe, C. V., and Pedlow, J. T., *J. Biol. Chem.*, 1936, **113**, 75.

[2] Barron, E. S. G., *Advances in Enzymology*, Vol. 3, 1943.

[3] Dorfman, A., *Physiological Rev.*, 1943, **23**, 124.

* Fresh blood of an infected rat was made free of the blood elements by centrifugalization and washing twice with a 0.9% sodium chloride solution. A required amount of the parasites was then lysed with water.

acid.[2,4]

TABLE II.
The Aldolase Activity of Lysed *T. equiperdum.*
The system contained 0.028 M $NaHCO_3$, 0.003 M Na_2CO_3, 0.015 M NaF, 0.015 M KCl, 0.008 M exose-diphosphate (added from the side arm), .4 cc of a lysed preparation (about 3 × 10^8 arasites) in a total volume of 1.8 cc in Warburg lasks; gas phase 5% CO_2-95% N_2; temperature 38.5°C; reaction time 30 min.; 0.2 cc of 100% richloracetic acid added to stop the reaction.

Fructose split,	20 min. alk. P. formed,
μg	μg
1140	201

The transformation of glucose into triose phosphates was shown by the formation of acid, fructose and 20-minute alkaline hydrolyzable phosphates from glucose and adenosine triphosphate (Table I). The aldolase activity may also be shown by measuring the disappearance of fructose 1, 6-diphosphate and the quantity of triose phosphates formed (Table II). The activity of 3-phosphoglyceraldehyde dehydrogenase is indicated by the production of acid in a system containing fructose 1, 6-diphosphate, diphosphopyridine nucleotide and arsenate. The rate of this reaction is not influenced by the presence of pyruvate. Sodium iodoacetate inhibits the reaction (Table III).

TABLE III.
Effect of Pyruvate and Iodoacetic Acid on 3-Phosphoglyceraldehyde Dehydrogenase in Lysed *T. equiperdum.*
The samples contained 0.026 M $NaHCO_3$, 0.005 M Na_2HAsO_4, 0.03 M NaF, 0.00037 M DPN, 0.005 M fructose 1,6-diphosphate (tipped in from the side arm after equilibration), 0.5 cc of a lysed preparation (about 2 × 10^8 parasites) in a total volume of 2.5 cc. 0.01 M Na-pyruvate or 0.0012 M Na-iodoacetate was added as indicated. Warburg manometers; gas phase 5% CO_2-95% N_2; temperature 37.5°C. The values are the amounts of CO_2 evolved in microliters.

Sample	Time of reaction 15′	30′	45′	60′
Control system	10.1	15.7	20.6	23.9
Arsenate plus pyruvate	12.0	19.3	25.0	27.7
Arsenate plus iodoacetate	3.8	4.2	4.6	5.9

TABLE IV.
The Activity of Adenosine Triphosphatase in Lysed *T. equiperdum.*
The samples contained 0.4 cc veronal buffer of pH 7.4, 0.1 cc 0.154 M KCl, 0.1 cc 0.154 M $NaHCO_3$, 0.1 cc 0.0032 M ATP, 0.1 cc of a lysed preparation (about 2 × 10^7 parasites), and 0.1 cc of 0.003 M $MgCl_2$, 0.015 M $CaCl_2$, or 0.015 NaF; 0.1 cc of 100% trichloracetic acid was added to terminate the reaction. Temperature, 39°C; time, 1 hour.

Sample	% of ATP split
With Mg	39.1
With Ca	11.2
With F	0.0
Without Mg, Ca, or F	9.8

Adenosine-triphosphatase activity is high in the preparation (Table IV).

Conclusion. Our experiments indicate that glucose is degraded into pyruvic acid through phosphorylative processes in a blood-free preparation of lysed *Trypanosoma equiperdum.*

---

[4] Speck, J. F., and Evans, E. A., Jr., *J. Biol. Chem.*, 1945, **159**, 71.